Journal of Embryology and Experimental Morphology

VOLUME 4 *March 1956* PART 1

PUBLISHED FOR THE COMPANY OF BIOLOGISTS LIMITED

OXFORD : AT THE CLARENDON PRESS

INFORMATION ABOUT THE JOURNAL

The main object of the Journal is to bring together work concerned with all the kinds of developmental process to be found in animals at any stage of their life-history. It will include work on embryology (descriptive, experimental, chemical, physiological), on regeneration and healing, on the developmental changes involved in functional adaptation, on growth, ageing, and other aspects of late morphogenesis. It will publish studies of gametes and gametogenesis where the work is not primarily of cytological interest; and studies of developmental genetics and of the physiology of reproduction where these fall more within its scope than within the usual scope of existing specialized journals. Papers on those aspects of protozoology which have a bearing on morphogenesis will be acceptable. Papers concerned with developmental processes in plants may be included if they link up with zoological aspects of morphogenesis.

The papers published will for the most part be accounts of finished pieces of research. Preliminary reports will not be published. Theses and very long papers are unlikely to be accepted at present. Theoretical and review papers may be published from time to time, as space allows. Contributions may be English, French, or German. Contributors wishing to use other languages should consult the Editor.

Journal of Embryology and Experimental Morphology is published quarterly. Subscription price £4. 4*s*. 0*d*. yearly for the four numbers; for this sum (*prepaid*) the Journal is sent Post Free to any part of the world. Separate numbers price 25*s*. Correspondence, except that about the subject-matter of the Journal which should be sent to the Editor, should be addressed to the Publisher:

GEOFFREY CUMBERLEGE
OXFORD UNIVERSITY PRESS, AMEN HOUSE, LONDON, E.C.4

On the Embryology of the Isopod *Irona*[1]

by S. GOPALAKRISHNAN NAIR[2]

From the University Zoology Laboratory, Madras

CONTENTS

[1] From a thesis approved for the degree of Master of Science of the Madras University.

[2] *Author's address*: Fisheries Research Laboratory, Port Blair, Andaman and Nicobar Islands, India.

[J. Embryol. exp. Morph. Vol. 4, Part 1, pp. 1–33, March 1956]

INTRODUCTION

ALTHOUGH Rathke (1834), Dohrn (1867), van Beneden (1869), and Roule (1889, 1890, 1891, 1894, 1896) have studied the embryology of Isopoda, the first detailed account and the one that is ordinarily quoted in text-books is that of Bobretzsky (1874) on *Oniscus murarius*. This work is informative in a general way, though the details of segmentation and germ layer formation are not accurate. Bullar's (1878) work on the parasitic isopods was largely influenced by the generalizations of Bobretzsky. Nusbaum (1891*a*, 1898) and McMurrich (1892, 1895) have contributed considerably to our knowledge of segmentation and post-mandibular growth in isopods but their accounts of the different fates of the germ layers left several problems of embryology unsolved. Goodrich's (1939) studies on *Porcellio* and *Armadillidium* were confined mainly to the origin and fate of the endoderm elements. Manton's (1928) paper on the development of *Hemimysis* serves as a landmark in the history of Crustacean embryology. A study of isopod literature reveals that the earlier workers did not take into account details regarding the structure and distinguishing features of mesoderm and endoderm. It is therefore possible, as Manton (1928) suggests with regard to germ layer formation of Malacostraca in general, that improved technique may yield different results.

The significance of the origin, differentiation, and fate of the mesoderm in the pre-antennulary segment was first described for *Hemimysis* and *Nebalia* by Manton (1928, 1934). Later Nair (1949) and Aiyer (1949) described it in the decapods, *Caridina* and *Palaemon*. The occurrence of the pre-antennulary mesoderm in isopods has not been recorded, though Bullar (1878) and Goodrich (1939) have sketched it without describing it as such.

It was therefore felt that a study of one of the parasitic isopods in the light of the recent work of Manton (1928, 1934) and Nair (1939, 1949) would add to our knowledge of Malacostracan embryology, especially if the process of gastrulation, the formation of the liver, the development of the pre-antennulary, thoracic and abdominal mesoderm, the formation of the heart, and the fate of the endoderm cells were investigated in detail.

The author wishes to express his deep indebtedness to Dr. S. M. Manton for revising the manuscript and offering her valuable suggestions. His grateful thanks are due to Dr. C. P. Gnanamuthu, Professor of Zoology, University of Madras, under whose kind guidance this work was done, and to the University of Madras for awarding a studentship during the first two years of the work.

MATERIAL AND METHODS

The following account of the embryology of *Irona* is based mainly on the eggs of *Irona robusta* and supplemented from a study of the eggs of *Irona far*. These forms, parasitic on *Hemirhamphus* and *Ptylosurus*, were collected when the host fishes were netted along the Madras coast, mainly during the months of October

and February (1949–52). The eggs in each brood were at the same stage of development, and numbered 275–350.

Eggs were removed from the brood pouch and fixed and embedded according to the procedure of Manton (1928). As the large quantity of yolk present at early stages interfered with sectioning, it was found necessary to remove as much of it as possible. Sections were cut at 8 μ and stained with iron haematoxylin and counterstained in eosin. The earlier stages of cleavage, after the appearance of the blastomeres on the surface of the egg, were followed by staining the egg in bulk with Delafield's haematoxylin and differentiating with acid alcohol, whereby the yolk alone appeared deep blue leaving the cytoplasm as whitish stellate patches with central dark nuclei.

For preparing whole mounts of the blastodiscs, eggs in which a blastoderm could be seen under the binocular were selected and the chorion removed. The bulk of the yolk was then removed from the blastoderm with a scalpel, and the yolk granules adhering to the lower surface were scraped off carefully with needles under a binocular microscope. The blastodiscs were then stained with iron haematoxylin and mounted in glycerine.

THE EGG

The egg of *Irona*, Fig. 1, when fresh is white in colour and oval in shape, measuring about 1·02 mm. in length and 0·90 mm. in breadth with a central nucleus, surrounded by a stellate mass of protoplasm, embedded in a semi-fluid yolk, and covered by a thin layer of peripheral protoplasm. It thus resembles the eggs of *Jaera*, *Asellus*, *Porcellio*, and *Armadillidium* (McMurrich, 1895), and *Hemimysis* and *Nebalia* (Manton, 1928, 1934). The extruded egg has only one enveloping membrane, the chorion, which is elastic and sticky. A definite vitelline membrane is not evident in the single-celled stage, but a thickening of the peripheral protoplasm noted after the third division probably represents this membrane.

SEGMENTATION OF THE EGG AND FORMATION OF THE GERMINAL DISK

The earliest stage examined shows eight blastomeres within the yolk just internal to the peripheral protoplasm, of which two are seen in Fig. 2. Since no blastomeres could be detected on the periphery of the egg prior to the eight-celled stage, it has to be inferred that the first three divisions were internal. These eight blastomeres emerge out of the yolk as stellate masses of protoplasm with deeply staining nuclei at one pole of the egg and arrange themselves in the form of a circle (Fig. 3).

In 16-, 32-, and 64-celled stages (Figs. 4, 5, and 6) the blastomeres were seen to migrate to the opposite pole. A group of 10–11 cells always moved ahead of the rest in the form of a circle, while the others were scattered in the region where

they first appeared. The 10–11 cells moving in a ring, after reaching the opposite pole, arrange themselves in a compact manner to form the rudiment of the blastoderm. Migration of the other cells follows subsequently (Fig. 7), and they all become incorporated into the blastoderm.

The cells of the early blastodisc are polygonal in shape, with prominent central nuclei, and rest directly on the yolk. Immediately below the blastoderm the superficial yolk is granular and contrasts with the compact globules seen in the bulk of the yolk inside. This change in the composition of yolk may be the first indication of the 'utilization of the yolk by the embryo by a hydrolysis of the complex lipoprotein' (Brachet, 1950). As development proceeds this granular yolk increases in quantity. When the blastoderm grows to about 479 μ in diameter there are no loose blastomeres, as all have taken part in the formation of the disk.

When fully formed the blastoderm exhibits three regions where the cells proliferate more rapidly than elsewhere, as can be seen by the greater number of mitotic figures (Fig. 8). Of these three regions the anterior two give rise to the future cephalic rudiments while the posterior one forms the blastoporal area which is demarcated from the rest of the blastoderm by a crescentic row of cells (Figs. 7 and 8 *c.r.*). Goodrich (1939) found a similar crescentic arrangement of cells delimiting the blastoporal area in *Porcellio*. This crescentic arrangement of cells leads to the horseshoe of ectodermal teloblasts which may be the direct successors of these blastoderm cells.

GASTRULATION

The studies of McMurrich (1895), Manton (1928), Goodrich (1939), and Nair (1939) show that gastrulation in Peracarida is by the immigration of cells from the blastoporal area towards the yolk. In *Irona* the blastoporal cells, delimited from the rest of the blastoderm by the crescentic row (Figs. 7 and 8), show a greater affinity for stains than the rest of the blastoderm. Cells migrate inwards from the posterior part of the embryo (Figs. 9–12) and leave gaps or small pits on the surface of the blastodisc, as seen by Manton (1928). A shallow depression, which is caused by the migration of cells into the interior, is the blastoporal homologue (Fig. 12, *bl.a.*). In slightly older embryos the depression fills up, probably by the multiplication of the adjacent cells. The internal cells arrange themselves below the blastopore as a plug (Fig. 13) projecting into the yolk, which can be compared to the 'bouchon blastoporique' of Sollaud (1923). Later stages show that this plug gives rise to the mesodermal head bands, the mesodermal teloblasts, the liver, and a portion of the endoderm. As the derivatives are both mesodermal and endodermal in character, this plug may be called the mesendodermal plug. The pre-antennulary mesoderm arises independently from the blastoporal mesendodermal mass (see p. 10).

In the embryos where the mesendodermal plug has become a few layers thick, two small groups of cells are formed at the anterolateral margins of the plug,

which grow so that the plug becomes V-shaped. This V-shaped mesoderm is characteristic of malacostracan development. In the initial stages the arms of the V are not conspicuous and are seen to be made up of a single row of cells. The arms appear more marked when rudiments of the naupliar appendages become distinguishable (Fig. 19).

When the V-shaped mesodermal band is being formed, a few isolated cells from the mesendodermal plug at the apex of the V degenerate (Figs. 14 and 15). These cells show certain cytological changes prior to degeneration. Their nucleoli become enlarged and globular, though no change is at first evident in the nuclear reticulum. When the nucleolar globules reach a certain size other small globules appear in the nuclei and the chromatin becomes scanty. Later the nucleolar globules as well as the others unite to form a large globule altering the shape of the nuclear membrane. This is followed by the rupturing of the nuclear membrane and the breaking up of the cells. The globules, the fragments of the nuclear matter as well as the cytoplasm of the cells, are left on the surface of the yolk and are stained deeply.

When the headbands are differentiated some of the cells at the antero-lateral margins of the mesendodermal plug become transformed into the mesodermal teloblasts (Fig. 17). The nuclei in these cells appear slightly larger than those of the surrounding cells and occupy a central position within the cytoplasm. The chromatin granules become arranged in the peripheral region of the nucleus. These features of the nucleus distinguish the mesodermal teloblasts from the rest of the mesendoderm cells. The mesodermal teloblasts are arranged in the form of two arcs with a space between, below the crescentic row of ectodermal cells (Fig. 16). Each group is made up of four cells placed close to each other. Since all of them appear together in the same stage and since no sign of their differentiation is noticeable in slightly earlier stages it is fair to presume that they all arise independently by the transformation of existing mesodermal cells and not as products of division of one cell as stated by Nusbaum (1891*a*) for *Ligia*.

When the mesodermal teloblasts are well differentiated, a few cells from the posterior apex of the V immigrate towards the yolk. These cells absorb yolk into vacuoles. Such a transformation is characteristic of endoderm cells, as described by Manton (1928, 1934) and Nair (1949). Immigration of large numbers of endoderm cells from an area behind the ectodermal teloblasts follows (Figs. 18 and 19). The endoderm cells formed from these two places are scattered over the yolk below the other tissues. A few cells in the mesendodermal plug do not join in the formation of either the naupliar appendages or the endoderm. They are found to give rise to the future liver rudiment (see p. 12).

Just after the mesodermal teloblasts are fully formed the ectodermal teloblasts appear by the transformation of blastoderm cells. Whole-mounts of blastoderms show practically no differentiation in the ectoderm cells till the rudiments of the headband of mesoderm are laid down and the mesodermal teloblasts are formed. In the earliest stage nineteen ectodermal teloblasts are differentiated simul-

taneously (Fig. 16). The ectodermal teloblasts closely resemble the mesodermal ones in cytological details. In Fig. 16, where all the nineteen ectodermal teloblasts are present, no more immigration of cells from the blastoporal area is seen. But when the teloblasts commence proliferation immigrations of ectodermal cells take place from two places on the blastodisc: (1) in front of the antennulary rudiments (Fig. 43), and (2) posterior to the teloblasts, to give rise to endoderm (Figs. 18 and 19).

The differentiation of the three primary germ layers which give rise to the various internal organs is complete once the teloblasts are fully differentiated, though the immigration of the endoderm cells is continued even up to a stage in which the rudiments of all the thoracic and abdominal segments are established. The history of blastoderm cells can be summarized as follows:

A. The ectoderm is formed from three primordia:
   1. The ectoderm of the naupliar region directly from the blastoderm cells anterior to the crescentic row.
   2. The ectoderm of the post-mandibular region from the ectodermal teloblasts.
   3. The telson ectoderm from the blastoderm cells lying behind the teloblasts.

B. The mesoderm is formed from two sources:
   1. The pre-antennulary mesoderm is formed independently by the migration of blastoderm cells anterior to the antennules.
   2. The mesendodermal plug gives rise to (*a*) the mesoderm of the naupliar appendages, (*b*) the mesoderm of the liver, and (*c*) the mesodermal teloblasts supplying the post-mandibular segments, and the telson mesoderm.

C. The endoderm is formed from two places:
   1. From the mesendodermal plug.
   2. From the blastoderm cells lying behind the ectodermal teloblasts.

THE NAUPLIUS

Korschelt & Heider (1899) state that 'in those cases in which the young animal is hatched at a later stage of development (Cladocera, Arthrostraca and most Decapoda), the nauplius stage is thrown back among the series of embryonic changes'. In *Irona* the nauplius is only a passing stage when the differentiation of the headbands of mesoderm and the formation of the rudiments of the appendages first become evident.

The headband after its differentiation shows three groups of cells (Fig. 19) along each arm of the V which on further growth form the mesoderm of the naupliar appendages. That of the antenna appears first, then that of the mandible, and lastly that of the antennule. The ectodermal cells overlying the naupliar mesodermal somites do not show any differentiation at this stage (Fig. 16), and the nauplius stage can only be recognized from sections.

POST-NAUPLIAR DEVELOPMENTAL STAGES

The two species of *Irona* show only minor variations from the nauplius to the newly hatched larva, such as the intensity of pigmentation of the body wall. The salient features of the post-naupliar stages of *Irona robusta* are shown in Figs. 20–32.

Two rounded cephalic lobes, two pairs of antennae, and a pair of mandibles are seen externally in Fig. 20. The teloblasts have divided in such a way that their products are arranged in transverse rows which are destined to give rise to the first five post-mandibular appendages. Two pairs of maxillae and the maxillipeds have made their appearance as small protuberances in Fig. 21. The teloblasts continue to be active. Four of the anterior thoracic legs are differentiated in Fig. 22. The teloblasts here have ceased activity and are not recognizable.

The stomodoeum forms on the ventral side as a small depression at the junction of the two pairs of antennae in Fig. 23. The dorsal organ appears on the dorsal side of the head as a whitish crescentic disk. The thoracic region shows all the seven segments but there are only six pairs of appendages; the last pair appears only after hatching.

The cephalic lobes migrate laterally (Fig. 24). The oral appendages appear as buds while the maxilliped and the thoracic appendages appear as finger-shaped outgrowths. Slight bulgings appear in the middle of the posterior margin of each thoracic leg. These were considered to be the embryonic rudiment of the exopodite of the leg by Nusbaum (1891*b*). The telson grows towards the ventral side concealing the proctodaeum from view.

The antennae, the maxilliped, and all the thoracic appendages show constrictions into joints in Figs. 25 and 29. The rudimentary exopodites disappear. The pleopods become bilobed, the outer lobes arising as buds. The uropods and telson are seen only in lateral views.

Ommatidia of the developing eyes are seen on the posterior margins of the cephalic lobes in Figs. 26 and 30. The antennae move towards the ventral side. The mandible develops a three-jointed palp which projects laterally. Distally the mandibles turn anteriorly like the cephalic appendages. The seventh thoracic segment is seen only in lateral view. The rami of the pleopods have become foliaceous.

The cephalic lobes grow together and fuse on the dorsal side forming a compact head (Figs. 27 and 31). The first and second antennae show the full number of joints, viz. eight and nine respectively as in the adult.

In Figs. 28 and 32 the embryo is ready to hatch. Hatching takes place inside the brood pouch, when the mother is attached to the host. The straightening of the appendages and the telson appears to be responsible for rupturing the chorion and liberating the larva.

The larva measures 2·45 mm. in length with a distinct rounded head, a large thorax, and a narrow abdomen (Fig. 33). All the body segments are now clearly

seen, but while metamorphosing into the adult the first two abdominal segments become tucked under the last thoracic segment when its appendages develop, thereby reducing the length of the abdomen. The appendages of the larva resemble those of the adult (Nair, 1950).

ORGANOGENY

Growth of the post-naupliar region

The development of the post-mandibular region in all Malacostraca so far investigated takes place by the activity of the teloblasts. In the Leptostraca, Mysidacea, most of the Decapoda and Stomatopoda, the formation of the caudal papilla during teloblastic growth appears to be a common feature, whereas in Peracarida, excepting the Mysidacea, no caudal furrow is formed and the products of the teloblasts grow over the yolk posteriorly and are responsible for the growth of the post-naupliar region of the embryo.

The formation of the teloblasts has already been described. The blastoderm of Fig. 18 has twenty-five ectodermal teloblasts more or less in a line. It would appear that the nineteen ectoteloblasts of the earlier stage (Fig. 16), which were arranged in a horseshoe, have been reinforced in number from the ectoderm cells. In all mitoses of the teloblasts the axes of the spindles lay parallel to the longitudinal axis of the embryo, and their products were in a line with them anteriorly. Therefore the new teloblasts could not have originated by the division of existing teloblasts. Cytological changes in the ectodermal cells lateral to the teloblasts have often been noticed in surface views of blastoderms, thereby strengthening the view that these additional teloblasts arise by a transformation of already existing ectoderm cells. Anterior to the row of twenty-five ectodermal teloblasts lie rows of narrow elongated cells, arranged so that each of the cells of one row is in front of a cell of the row behind. This linear arrangement suggests that these cells are the descendants of the ectodermal teloblasts, as in other Malacostraca. The characteristic arrangement in rows makes it easy to distinguish the teloblastic from the original naupliar area. The straightening of the horseshoe of ectodermal teloblasts is due to the increased growth in size of the cells in the median region as suggested by McMurrich (1895). The mesodermal cells continue to be eight in number as they were in Fig. 16.

A longitudinal section through the embryo (Fig. 19) shows that while the ectodermal teloblasts have given rise to ten rows of descendants the mesodermal teloblasts have produced only seven. Computing the number of divisions which have yielded these seven rows, it is probable that only seven divisions of the original teloblasts in both rows have taken place, but that the first three rows of ectodermal descendants have divided again, so that for each mesodermal row there are two rows of ectoderm. Manton (1928) and McMurrich (1895) found this relation to be a constant feature in the formation of each post-naupliar segment.

Longitudinal sections of the differentiating telson of this stage (Fig. 34) show mesoderm cells arranged in three to four rows just behind the middle four mesodermal teloblasts. These cells go to the formation of the telson mesoderm while the blastoderm cells lying behind the teloblasts give rise to the ectoderm of the telson (Fig. 34).

The identity of the ectodermal and mesodermal teloblasts disappears when the sixth abdominal segment is formed. In the present study the author has not seen any evidence of the formation of a seventh abdominal segment.

Gut

Unlike the mysids, amphipods, and decapods, where the endoderm contributes towards the formation of the mid-gut, the entire gut in *Irona* is formed from ectodermal cells without the endoderm contributing towards its development, as in the other isopods *Porcellio* and *Armadillidium* (Goodrich, 1939).

The first part of the digestive tract to be formed is the stomodoeum, followed immediately by a proctodoeum. A small invagination of ectoderm cells between the two pairs of antennae in the mid-ventral region is seen in Fig. 35. This stomodoeal invagination becomes deeper, enlarging ventrally and posteriorly into a flask-shaped sac (Fig. 36). The proctodoeum is formed as an invagination from the blastoderm cells, which have been moving backwards *pari passu* with the growth in length of the embryo. This invagination begins from the region where the telson meets the last abdominal segment (Figs. 38 and 39). As growth proceeds the mouth becomes narrower, the long constricted part of the stomodoeum is destined to become the oesophagus while the rest forms the stomach. The walls of the stomodoeum are thicker on the dorsal and ventral sides but thinner at the posterior basal aspect. In Fig. 30 the proctodoeum has pushed forwards as far as the third abdominal segment.

The stomach assumes a triangular shape in Fig. 37, with a thicker ventral wall. The inner surface of this wall gives rise to three prominences, the rudiments of the gastric mill. At the angle opposite to this side of the triangle the cells increase in number and this part of the stomach appears to be pulled dorsally. Sections of later embryos suggest that this is due to the insertion of the pre-antennulary mesodermal muscle strands. It is also probable that owing to this pull the side of the stomach with the rudiments of the gastric mill becomes erected as in Fig. 32. At this stage the proctodoeum has extended forwards as far as the third thoracic segment. The wall of the proctodoeum, which had 1–2 rows of narrow elongated darkly staining cells near the opening, now becomes 2–3 cells thick. It has a distinct mesodermal investment over it. When the proctodoeal invagination enters the thorax it acquires a small cavity and broadens slightly, the wall being made of only one row of rounded cells.

In Figs. 32 and 37 the three rudiments of the gastric mill have become more pronounced, the middle one being the largest. The oesophagus has elongated, shifting the stomach backwards into the second thoracic segment. By the time

the larva (Fig. 33) is formed, the proctodoeum has pushed its way into the second thoracic segment and makes contact with the stomach dorsal to the gastric mill rudiments where the stomach is one cell thick. Continuity is established by the absorption of the two-cell thick septum. The three lobes of the liver (see p. 13) reach the ventro-lateral part of the stomach close to the gastric mill rudiments (Fig. 33). Therefore the forward extension of the proctodoeum has to pass between the liver lobes and the yolk before it can reach the stomach wall. Thus the gut is formed entirely by the stomodoeal and proctodoeal invaginations and the endoderm does not enter into its formation.

Dorsal organ

By the time the embryo reaches the stage shown in Fig. 22 a group of free ectoderm cells dorsal to the cephalic lobe on the median side of the head appears as a whitish circular patch and forms the rudiment of the dorsal organ. The cells of this group are elongated radially with prominent rounded nuclei. A slight invagination at the posterior end (Fig. 40) transforms this group of cells into a bag-like structure.

When the invagination is complete the cuticular covering of the cells within the sac becomes thicker and corrugated as in Fig. 41. This corrugated cuticle is at first seen very close to the cells of the sac or almost touching them. The pre-antennulary mesodermal strands become attached below the dorsal organ at this stage. Later, the cavity of the sac enlarges and the cuticular lining appears roughly in the centre of the cavity with a space separating it from the cell layer. A narrow ridge originating from the posterior end of the opening of the sac runs towards the postero-ventral floor of the sac, dividing the cavity into two at the extreme posterior end (Fig. 42). The nuclei of the cells then become elongated and their cytoplasm drawn out. The yolk immediately below the dorsal organ is made up of smaller globules which stand out in contrast to the solid bulk of yolk mass seen in the centre of the embryo. Very often globules of yolk lie between and at times inside the cells. This relation and distribution of the yolk suggests that the dorsal organ plays a part in the absorption of the yolk.

Before the larva hatches the ectoderm cells grow towards the dorsal side and form a layer, separating the dorsal organ from the yolk in which it was immersed. In the larva, the dorsal organ is raised and during the subsequent moult it is cast off.

Pre-antennulary mesoderm

When the teloblasts have commenced active proliferation (Fig. 18) a group of 6–8 cells of the blastoderm lying between the optic rudiment and the antennule in a line with the rest of the naupliar appendages sinks into the yolk to form the rudiments of the pre-antennulary mesoderm (Fig. 43). The migrating cells have narrow attenuated ends towards the outside and swollen basal portions towards the yolk, and are strikingly unlike the surrounding ectoderm and mesodermal

cells in their prominent nuclei and darkly staining granular protoplasm. No difficulty was experienced in recognizing these cells in *Irona*, in contrast to *Hemimysis* and *Nebalia* (Manton, 1928, 1934), because of their slightly larger number and their contrasting staining reactions.

The group of pre-antennulary mesoderm cells has multiplied to form a solid mass of cells with ectodermal connexions in Fig. 44 and Diagram A, 1. A coelomic cavity is formed in the pre-antennulary mesoderm by the time the stomodoeum invaginates between the antennules and the antenna in the midventral

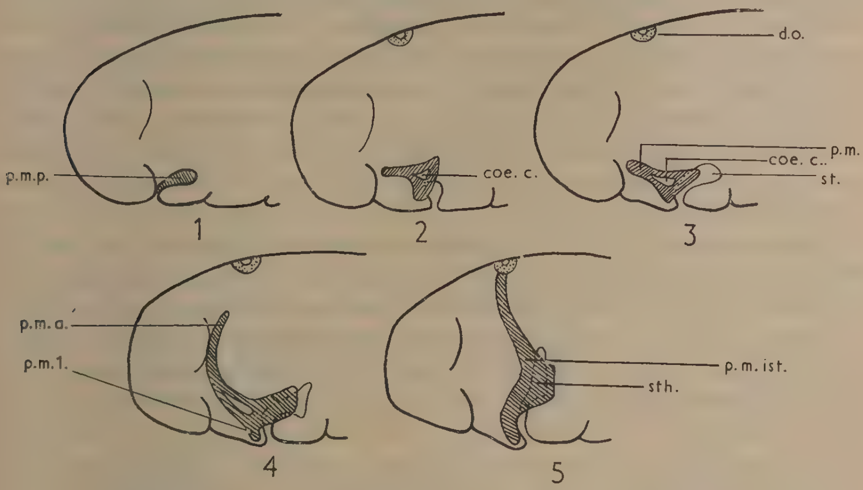


DIAGRAM A. Development of the pre-antennulary mesoderm. Key on p. 22.

region. Prominent changes in the pre-antennulary somite are noticed after the formation of the stomodoeum. The cells loose their ectodermal connexion and the two mesodermal blocks shift into the interior and come to lie obliquely, one on either side of the stomodoeum, their cavities enlarging. Later (Fig. 27), the cells of the posterior sac-like parts of the somite multiply in such a way that they give rise to two solid strands (Diagram A, 2). The ventral one grows towards the labrum, and from either side contributes towards the labral musculature. The other strand applies itself to the lateral walls of the stomodoeum (Diagram A, 3 and 4). The cavity of the somite develops only towards the stomodoeal branch and not towards the labrum.

The stomodoeal branch from each somite grows towards the dorsal side, the pair forming narrow solid divergent strands, the mesodermal cavity being confined to the region of the stomodoeum (Fig. 45). The broad ventral portion of the mesodermal mass, which first became attached to the stomodoeum, grows and meets its counterpart on the opposite side and forms an investment round the foregut. The divergent free ends of the blocks grow upwards along the inner side of the cephalic lobe and become attached dorsally to the tergum of the first

thoracic segment and then extend to the lateral margins of the dorsal organ (Diagram A, 4 and 5). Later still they grow under the anterior region of the dorsal organ, meet medially, and between them give rise to the lumen of the dorsal aorta (Fig. 46). The coelomic cavity becomes obliterated when the strands reach the dorsal organ and are converted into muscles.

As the dorsal organ grows and shifts above the surface of the embryo the pre-antennulary strands also become pulled upwards and with them the stomodoeum (see p. 9). During this process the curved mesodermal strands are straightened. The stomodoeal part of the pre-antennulary mesoderm gives rise to the circular muscles of the oesophagus and the muscles of the gastric mill, while the dorsal strands which suspend the stomodoeum at this stage later give rise to the dorsal mucles of the stomach. Thus from the pre-antennulary mesoderm are derived the labral muscles, the anterior end of the dorsal aorta, and the muscles of the gastric mill as well as the dorsal muscles of the stomach.

Naupliar mesoderm

The formation of the headband of mesoderm in the form of a V and its division into three pairs of somites at the nauplius stage has already been described. When the naupliar appendages become prominent, their mesoderm is derived from these solid somites which ultimately form the core of muscles. Sections of the naupliar region at different stages show that the headband is practically all used up in the formation of the naupliar appendages.

Liver

The liver has an undoubted endodermal origin in *Nebalia* and some other Malacostraca (Manton, 1934), but it was Manton (1928) who first described the development of the liver in *Hemimysis* from the mesoderm, unlike Bobretzsky (1874), Nusbaum (1886), Reinhard (1887), Roule (1895), McMurrich (1895), and Goodrich (1939), who believed that the liver had an endodermal origin. Nair (1939) described the liver in *Mesopodopsis* as originating from the naupliar mesoderm. In the development of the liver from the mesoderm, *Irona* resembles *Hemimysis* and *Mesopodopsis*.

In a median longitudinal section of a stage slightly earlier than Fig. 20, a group of about 7–10 mesoderm cells is noticeable in the maxillulary segment. These cells are characterized by their compactness and length as well as their darkly staining protoplasm. This is particularly clear when stained with haematoxylin. Sections of later stages (Figs. 21 and 22) show that this group of cells is the liver primordium. Since the products of the mesodermal teloblasts are arranged in rows one behind the other, and since this liver primordium occurs as a group of mesoderm cells distinct from the teloblastic products in the maxillulary segment, it is inferred that this group has originated from the apex of the V-shaped head mesoderm band formed at the naupliar stage.

As growth proceeds (Fig. 22) the development of the nerve ganglia of this

segment causes the division of the original central liver rudiments into two groups, one on either side (Fig. 47). In the next stage (Fig. 23) each group moves towards the lateral margins of the embryo and comes to lie as a plate between the yolk and the ectoderm. While so shifting the edges curl dorsally (Fig. 48), the rudiments becoming saccular. The isolated mesoderm cells often seen in the vicinity of the liver give rise to the connective tissue of the liver. Sections of the liver at this stage show that each rudiment grows in length and extends posteriorly to the maxillary segment.

In Fig. 24 each liver rudiment has its open end directed towards the dorsal side as before. The opening contracts slightly, and in Fig. 25 the primordium shows indications of three lobes (Fig. 49). These primordial lobes (Fig. 50) become differentiated into the three liver lobes of the adult. The growth of the liver in relation to other organs can be seen in Figs. 29–33. The sac-like primordium on either side, with its small opening dorsally facing the yolk, grows posteriorly in such a manner that, while the opening remains anterior, the three lobes extend backwards as far as the last thoracic segment. In this backward growth the three lobes become elongated into three hollow tubes, the anterior undivided part being still open towards the yolk (Fig. 32). Later the paired anterior openings of the liver primordium meet the wall of the stomodoeum as it grows backwards, and then open into its lumen (Fig. 33).

Histological changes in the liver cells during development. In Figs. 47–49 the walls of the liver sac are made up of compact uniform cells with no vacuoles, arranged in 3–6 rows. When the differentiation of the liver lobes starts minute vacuoles of about 1 μ diameter appear scattered in the protoplasm, and cell limits are not here discernible (Fig. 50). These vacuoles gradually enlarge and unite with each other to give larger vacuoles (Fig. 51) which are formed towards the interior of the lobes, the nuclei thus becoming displaced towards the periphery.

Sections taken along the length of the liver lobes show that in the most posterior region the vacuoles are large and are surrounded by a ring of nuclei while in the more anterior region where the cells are younger the vacuoles are small and not definitely arranged in relation to the nuclei.

Histological studies of later embryos show that these tubes help in the absorption of yolk, which in the absence of an endodermal mid-gut has to be utilized by different organs. Sections (Fig. 53) of the larva (Fig. 33) wherein the peripheral arrangement of the nuclei of the liver lobes is complete, show that the yolk surrounding the outer side of the lobes is broken up into small fragments. Figs. 52 and 53 show fragments (*y.b.*) of yolk very closely contiguous with the protoplasm of the liver lobes in which the nuclei are embedded. Here and there in the more posterior region of the tubes finer yolk particles (*y.p.*) are seen to lie between the nuclei, penetrating into the interior. Several such lines of small particles lead towards the lumen of the tube, where they lose their identity and appear to be absorbed. If this close association of the yolk outside the liver

lobes with the protoplasm of the liver cells is not interpreted as a process in the absorption of the yolk, it may suggest that the liver functions in the same way as the endodermal cells which surround the yolk during the formation of the mid-gut of decapods and mysids. Previous workers such as Bobretzsky (1874), Roule (1889), Bullar (1878), and Goodrich (1939) who studied the embryology of isopods may have been led to such an incorrect interpretation, in their search for uniformity with the rest of the Crustacea (see pp. 19–20).

When the larva hatches from the egg membrane, the protoplasm of the liver is highly granular, doubtless due to the absorption of yolk. The granules now do not stain with haematoxylin or eosin as did the yolk particles. It may be inferred that as the yolk fragments split and move towards the lumen of the tube they become chemically changed or absorbed.

Bullar (1878) in his sketches of sections of liver of *Cymothoa* indicates liver cells with vacuoles arranged in a similar manner. Unfortunately he has not interpreted the arrangement. It is very likely that in other cymothoans also the digestion of yolk by the liver cells may occur as in *Irona*.

Trunk mesoderm

When the rudiments of the naupliar appendages are differentiated from the arms of the V-shaped mesoderm band, and the liver rudiments are differentiated from the apex of the V, the eight mesodermal teloblasts lying below the ectodermal teloblasts give rise to sets of eight daughter cells in rapid succession. The eight cells of each set form the foundation cells of the mesoderm of each of the sixteen segments of the entire post-naupliar region (Fig. 54). Each segment at first possesses two rows of ectodermal cells. These as well as the foundation cells of the mesoderm divide rapidly. The mesodermal descendants arrange themselves in definite patterns as can be seen in Figs. 55 and 56, which are transverse sections through the metanaupliar region. When the eight foundation cells divide they give rise to an anterior row of eight cells, four of which after moving laterally to either side of the nerve-cord divide again and form a narrow band of eight cells (Fig. 56). The posterior eight cells also shift to either side of the nerve-cord. These cells also divide a second time, and their products shift so that four cells are crowded into a central group with a pair on either side, as seen in Fig. 55. Thus in each segment on either side of the nerve-cord there is an anterior strip of four cells closely set, and behind them eight cells arranged in three groups.

In Fig. 23 the number of cells forming the three posterior groups of cells within every segment has increased considerably although their arrangement is not altered. On each side of the nerve-cord three blocks of cells, the ventral, middle, and dorsal mesodermal blocks, can be seen (Fig. 57). In Fig. 24 the limb rudiment makes its appearance in each segment, and a block of cells enters the ectodermal dilatation to form the limb mesoderm which ultimately supplies the limb muscles. The dorsal block of mesoderm in each segment develops a cavity

(Fig. 57) and by further development gives rise to the heart, the pericardium, and the dorsal muscles in the posterior trunk segments, and to the dorsal aorta and muscles in the anterior eight post-mandibular segments. The ventral mesodermal block undergoes comparatively slower growth and gives rise to the ventral longitudinal muscles.

Meanwhile the closely set cells of the narrow band lying in the anterior part of each segment divide and increase in size. The inner end of this strip now shows a clear ectodermal connexion. Since there are no limb buds in this region, this strip divides into two, a ventral and a dorsal block. The former gives rise to the ventral muscles and the latter to the dorsal muscles, the heart, and the pericardium, in close relation with the posterior sets of mesoderm cells in the same

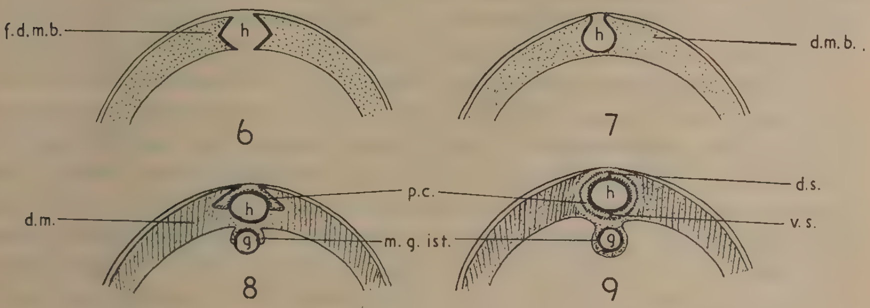


DIAGRAM B. Development of the heart. Key on p. 22.

segment. The cells of each segment multiply and differentiate in close relation to the cells of the segment in front and behind. As the heart is formed only in the abdominal and last two thoracic segments, the course of development of the dorsal blocks of cells in the maxillulary to sixth thoracic segment differs from that in the hinder region.

(A) *Development of the heart and pericardium.* Development of the heart first begins in the last abdominal segment and proceeds anteriorly to the sixth thoracic segment. All details of development of the heart can be seen in the same embryo (Fig. 31). The stages in the formation of the heart and pericardium are shown in Figs. 58 and 59 and Diagram B. The dorsal mesodermal blocks, which have the beginnings of the coelomic cavities, rapidly grow upwards as solid strips, the cavities being confined to the lower part of the mesodermal blocks. When the somites approach the mid-dorsal region from opposite sides each bifurcates into two branches (Diagram B, 6). The ventral branches unite with their fellows from the opposite side, while the dorsal branches attach to the dorsal body wall so that a haemocoelic space (*h*) is enclosed between the forks of the mesodermal blocks on either side, the dorsal ectoderm forming the roof (Fig. 58 and Diagram B, 7). This space so enclosed develops into the lumen of the heart.

Meanwhile the coelomic cavity within each mesodermal block attains its maximum size and extends upwards to about half the length of the block as a narrow elongated cavity with a thin inner wall made up of 1–2 rows of cells and a slightly thicker outer wall of 3–4 rows of cells. This cavity becomes obliterated by the multiplication of the cells on either side by the time the lumen of the heart is well established.

The rudimentary heart now develops a mesodermal roof by the multiplication of the cells from its sides (Diagram B, 8). By the time the roof of the heart is complete the bulk of the mesodermal strands differentiate into muscles dorso-laterally and two spaces appear on either side of the heart (Diagram B, 8). Each cavity is roughly triangular in cross-section and is roofed by the ectoderm above and flanked by the heart on one side and the differentiating dorsal muscles on the other side. These are the beginnings of the pericardium. The proctodoeum now lies below the heart. During further growth the pericardial cavity extends towards the ventral side encircling the heart laterally and ventrally. Cells from the ventral wall of the heart multiply and spread round the dorsal, lateral, and later the ventral surface of the gut (Fig. 59 and Diagram B, 8 and 9). Thus the ectodermal alimentary canal acquires a mesodermal investment. During the formation of this investment the pericardial cavities from opposite sides meet medially below the heart, leaving a thin septum dorsal to the proctodoeum. The pericardial wall extends more slowly towards the dorsal side of the heart, and then the heart sinks away (Fig. 59 and Diagram B, 9) from the ectoderm with which it retains its connexions by thin strands of mesoderm cells at irregular intervals along the mid-dorsal line. A compact pericardial wall is defined by the time the dorsal longitudinal muscles are fully formed.

(B) *Development of the dorsal aorta.* As the dorsal aorta represents the continuation of the heart anterior to the sixth thoracic segment, its development is essentially similar to that of the heart. The forks of the dorsal mesodermal blocks are smaller, the lumen of the aorta being formed by their union. Thus a median dorsal aorta is established from the sixth thoracic segment up to the cephalic region. The anterior tip of the aorta is formed from the pre-antennulary mesodermal strands (see p. 12).

Sections through the sixth thoracic segment of the stage in Fig. 28 show two constrictions dividing the lumen of the dorsal aorta in this region into three inter-communicating spaces at the junction of the aorta and the heart. The median space forms the dorsal aorta and the others form the beginnings of the lateral aortae which are completed after hatching.

Endoderm

The role of the endoderm cells in the formation of the liver in isopods has been emphasized by various investigators (Bobretzsky, 1874; Roule, 1891, 1894; Nusbaum, 1891*a*; and Goodrich, 1939). Since the results of the present investiga-

tion show that the liver is mesodermal in origin, the formation and fate of the endoderm cells were traced with special care in *Irona*.

Endoderm cells are seen to originate from two places: (1) the earliest endoderm cells from the mesendodermal plug (see p. 5), and (2) the later endoderm from the blastodermal cells posterior to the ectodermal teloblasts after the latter become arranged in a straight line (Fig. 23). Since the majority of the cells in the mesendodermal plug are used up in the formation of the headband of mesoderm, the mesodermal teloblasts, and the liver, only a few cells remain to contribute to the endoderm.

Isolated cells of the blastoderm posterior to the teloblasts migrate inwards and absorb yolk. These cells as well as the earliest endoderm cells form a thin endodermal sheet which extends forwards over the yolk below the mesoderm. This process continues to the stage of Fig. 22, where the endoderm forms a lining separating the yolk from the germinal disk which lies ventral to it. In Fig. 23 the endoderm cells grow towards the lateral and dorsal sides of the yolk forming an endodermal covering, namely, the yolk membrane. Sections of the yolk membrane (Fig. 34, *y.m.*) show the presence of many thin elongated cells which have cast off their yolk as well as many others which retain yolk to varying degrees. This suggests that some of the cells, after having absorbed the maximum quantity of yolk into their vacuoles, separate from it, as in other Malacostraca.

In Fig. 29 the yolk membrane is fully formed around the yolk and a number of cells are seen scattered in the yolk at varying distances from the yolk membrane. Their appearance and distribution show that they have separated and wandered into the yolk from the yolk membrane. These are undoubtedly vitellophags. Their protoplasm is spread round the nucleus in the form of radiating strands which penetrate into the yolk. This migration of the vitellophags into the yolk occurs from all sides of the yolk membrane, and while wandering the vitellophags are seen to divide and increase in number. These vitellophags help in the 'splitting of the yolk balls' (Bobretzsky, 1874) before they disintegrate. The presence of a large number of these vitellophags below the differentiating liver lobes suggests that they may be playing an important part in the transformation of the yolk in this area and thus facilitate the growth of the liver through the yolk.

As growth proceeds (Figs. 31 and 32) some of the vitellophags disintegrate in the interior of the yolk. The process of disintegration conforms to the usual course except for certain features. The nucleus shows 3 or 4 lobes (Fig. 60). Later the nuclear reticulum appears loose and the chromatin scanty as found in disintegrating vitellophags described by previous workers. The number of vitellophags decreases after the stage shown in Fig. 32. Only a few are seen scattered in the yolk of the larva and even these show a tendency to degenerate. Thus the endoderm cells do not contribute to the formation of any permanent organ in *Irona* (see p. 19).

DISCUSSION

The eggs of isopods were considered telolecithal by Dohrn (1867), Bobretzsky (1874), Roule (1889, 1890, 1891), and Nusbaum (1886), who did not study the egg in the unsegmented and early stages of cleavage. Reinhard (1887) first described the centrolecithal nature of the isopod egg in *Porcellio* and it was confirmed by McMurrich (1892, 1895). The protoplasmic connexions between the central and peripheral cytoplasm described by McMurrich for *Jaera* are not seen in the oocytes and the extruded eggs of *Irona*.

In the isopods, *Jaera, Asellus, Porcellio, Armadillidium* (McMurrich, 1895), *Porcellio, Armadillidium* (Goodrich, 1939), and *Cymothoa* (Bullar, 1878), the only membrane described is the chorion. In *Irona* also there is only one membrane, the chorion, which is secreted by the follicular cells. But during later stages a second membrane is secreted by the peripheral protoplasm of the embryo, making its appearance at the eight-celled stage. The late appearance of a vitelline membrane has been observed by van Beneden (1869) for *Asellus* and Terao (1929) for *Panulirus*.

The early cleavages of *Irona* resemble those of *Porcellio* and *Armadillidium* (McMurrich, 1895), the mysids (Manton, 1928; Nair, 1939), and *Nebalia* (Manton, 1934) in that these divisions take place within the yolk but do not affect it. The blastomeres appear on the surface of the egg in *Irona* at the eight-celled stage, but in *Porcellio* not till the thirty-two-celled stage, and in mysids a completed layer within the yolk is formed before the blastomeres appear on the surface. In the early appearance of the blastomeres at the periphery *Irona* shows more resemblance to *Nebalia*, in which the cells reach the surface of the egg at the four-celled stage.

A tendency for a group of 10–11 cells to move ahead of the others in a circle from their pole of origin to the opposite pole is an unusual feature. The present study of *Irona* shows that the egg and embryos are less determinate than are those of *Jaera* (McMurrich, 1895); the blastoderm cells in *Irona* are so alike that the cells which are destined to become the mesoderm and endoderm cannot be recognized in the early stages.

The type of germinal disk formation in *Irona* is more like that of *Leander* (Sollaud, 1923) than that of a mysid (Manton, 1928; Nair, 1939). In *Hemimysis* the germinal disk starts as a transverse band which later becomes V-shaped. In *Nebalia* (Manton, 1934) the germinal disk is at first pear-shaped, then it divides anteriorly, becoming V-shaped and later U-shaped as in Decapoda. Though the germinal disk of *Irona* is pear-shaped to begin with as in *Nebalia*, it does not become either a V or a U. In *Irona* the mesendodermal plug gives rise to mesoderm from the anterior and endoderm from the posterior regions, a spatial relationship characteristic of Malacostraca (Manton, 1928).

The entire post-mandibular region in *Irona* is formed by the activity of the teloblasts as in other Malacostraca (Manton, Nair, and others). The absence of

a caudal furrow may be due to the fact that the growth of the post-mandibular region is relatively slow and the elongated egg gives the embryo ample room for developing its entire length on the surface without folding inwards.

Irona resembles *Hemimysis* (Manton, 1928) in the prolonged endoderm formation from the blastoderm cells situated behind the teloblasts. Differentiation of the vetellophags occurs rather late and, unlike a mysid, all the endoderm cells or vitellophags disintegrate without contributing towards the formation of any permanent tissue of the isopod. In *Porcellio* and *Armadillidium* Goodrich (1939) has described a 'clumping' of 4–6 cells of the serosal nuclei which he marks clearly inside the yolk in his figures. These, from their position, must be considered to be vitellophags. A casual study of *Irona* may also suggest such a condition, but careful examination will show that there is no real grouping of cells. The nucleus of each vitellophag becomes lobed prior to its breaking up, and in this condition the nucleus gives the appearance of 4–6 nuclei coming together.

The discovery of the pre-antennulary mesodermal somite, first reported by Manton (1928) in *Hemimysis* and then in *Nebalia* (1934) has since been recorded in *Mesopodopsis*, *Squilla*, and *Caridina* (Nair, 1939, 1941, 1949) and *Palaemon* (Aiyer, 1949). The two species of *Irona* investigated here conform to the general features outlined by Manton (1928). In *Irona* a coelomic cavity is formed as in *Hemimysis* but is obliterated before the mesoderm strands reach the dorsal organ. The second cavity described for *Hemimysis* does not appear in *Irona*. The pre-antennulary mesodermal somites move between the cephalic lobes to reach the dorsal side of the animal as in *Nebalia*. This course of the somites between the cephalic lobes may be due to the lesser quantity of yolk present in the head at this stage.

The liver is mesodermal in origin in *Irona* as well as in the mysids (*Hemimysis*, Manton, 1928, and *Mesopodopsis*, Nair, 1939). Manton (1928) has defined the features which distinguish endoderm cells from those of the other two germ layers in *Hemimysis*, and these features can only be seen with good fixation. Not being able to distinguish the endoderm from other tissues, the older writers, Dohrn (1867), Roule (1891, 1894), Reinhard (1887), and Nusbaum (1891*a*, 1898) considered the liver to be endodermal. Even Goodrich (1939) has not recognized these cytological differences, and has described the liver in *Porcellio* and *Armadillidium* as endodermal. He failed to note both the origin of the liver from the naupliar blastoporal mesoderm, and its mesodermal character.

Diagnosis of the endoderm is essential in following the origin and formation of the gut. In *Irona* the yolk membrane is the homologue of the endodermal mid-gut of mysids and decapods, but it does not contribute to the formation of the gut as in mysids (Manton, 1928; Nair, 1939), decapods (Sollaud, 1923; Nair, 1949; Aiyer, 1949), and the Leptostraca (Manton, 1934). Manton (1928) suggested that if the proctodoeum were to become longer in a form like *Hemimysis*, then many of the yolk cells would not be included in the formation of an endo-

dermal mid-gut and would degenerate. As shown above, the ectodermal proctodoeum of *Irona* extends forward so completely that the endoderm cells are all excluded.

Since the gut of *Irona* is ectodermal, and the endodermal yolk membrane is broken up and functionless in the later embryonic stages, some device for yolk absorption appears to be necessary. Evidence has been given to show that the liver performs this function. Manton (1928) found alterations in the yolk taking place opposite the future lumen surface of the liver lobes, and this presumably occurs as a result of their presence. In *Nebalia* also yolk is absorbed from the lumen surface of the liver lobes (Manton, 1934). *Irona* is remarkable in the yolk absorption taking place from the future external surface of the liver lobes. Yolk particles are found also in the dorsal organ which therefore may assist in absorption of yolk. The capacity of the adult liver cells to absorb fat and other food substances has been observed by McMurrich (1898), Murlin (1902), Nusbaum (1917), Nicholls (1931), and Chandy (1938). McMurrich (1898) even suggests that in parasitic isopods 'the intestine, not being absorptive but merely serving as a passage for the extrusion of undigested material, is, in *Bopyrina*, exceedingly reduced, while the digestive and absorptive liver pouches are enlarged'. From the present study it appears that the absorptive capacity of the liver cells is a marked feature of *Irona* in the embryonic stages.

As in other Crustacea the heart and dorsal aorta are formed from a fork of the dorsal mesodermal blocks. The pericardial floor is differentiated at a late stage from the dorsal mesoderm after the formation of the heart in *Irona*, unlike *Hemimysis* (Manton, 1928) wherein it is differentiated at a very early stage.

SUMMARY

1. The structure of the egg is described.

2. Segmentation of the egg is followed from the eight-celled stage up to the formation of the blastoderm. A migration of blastomeres from one pole of the egg towards the opposite has been observed and in the course of this migration a circle of 10–11 cells moves in front of the others.

3. Gastrulation takes place by immigration. A V-shaped mesodermal head-band is differentiated which gives rise to the rudiments of the naupliar appendages after gastrulation is completed.

4. The entire post-naupliar region of the body is formed by the activity of ectodermal and mesodermal teloblasts. No caudal furrow is formed.

5. The gut is ectodermal, formed by the union of the stomodoeum and a long proctodoeum.

6. An ectodermal dorsal organ forms in early development and persists throughout embryonic life. The pre-antennulary mesodermal strands become attached to this organ.

7. The origin, development, and derivatives of the pre-antennulary mesodermal somites are described.

8. The origin of the liver is traced from the naupliar mesoderm. The probability that the liver plays an important part in yolk absorption is discussed.

9. The development of the trunk mesoderm and an early grouping of cells into segmental and intersegmental regions are described.

10. The formation of the heart, pericardium, and dorsal aorta is described.

REFERENCES

AIYER, R. P. (1949). On the embryology of *Palaemon idae* Heller. *Proc. zool. Soc. Beng.* **2**, 101–47.

BENEDEN, E. VAN (1869). Recherches sur l'embryogénie des Crustaces. 1. *Asellus aquaticus. Bull. Acad. Belg. Cl. Sci.* **28**, 54–57.

BOBRETZSKY, N. (1874). Zur Embryologie des *Oniscus murarius. Z. wiss. Zool.* **24**, 179–203.

BRACHET, J. (1950). *Chemical Embryology*. Interscience Publishers, New York and London.

BULLAR, J. F. (1878). On the development of the parasitic Isopoda. *Phil. Trans.* **169**, 505–21.

CHANDY, M. (1938). The histology and physiology of the intestine and hepato-pancreas of two Isopods, *Ligia exotica* Roux and *Armadillio elevatus* Verhoeff. *J. Asiat. Soc. Beng.* **4**, 1–16.

DOHRN, A. (1867). Die embryonale Entwicklung des *Asellus aquaticus. Z. wiss. Zool.* **17**, 221–78.

GOODRICH, A. L., Jr. (1939). The origin and fate of the endoderm elements in the embryogeny of *Porcellio laevis* Latr. and *Armadillidium nasutum* B.L. (Isopoda). *J. Morph.* **64**, 401–26.

KORSCHELT, E., & HEIDER, K. (1899). *Textbook of the Embryology of Invertebrates.* London. Vol. 3.

MANTON, S. M. (1928). On the embryology of a mysid Crustacean, *Hemimysis lamornae. Phil. Trans.* B, **216**, 363–456.

—— (1934). On the embryology of the Crustacean *Nebalia bipes. Phil. Trans.* B. **223**, 163–238.

MCMURRICH, J. P. (1892). The formation of the germ layers in the Isopod Crustacea. *Zool. Anz.* **15**, 271–5.

—— (1895). Embryology of the Isopod Crustacea. *J. Morph.* **11**, 63–154.

—— (1898). The epithelium of the so-called midgut of terrestrial Isopods. *J. Morph.* **14**, 83–108.

MURLIN, J. R. (1902). Absorption and secretion in the digestive system of land Isopods. *Proc. Acad. Nat. Sci., Philad.* **54**, 284–359.

NAIR, K. B. (1939). The reproduction, oogenesis and development of *Mesopodopsis orientalis* Tatt. *Proc. Indian Acad. Sci.* **9**, 175–223.

—— (1941). On the embryology of *Squilla. Proc. Indian Acad. Sci.* **14**, 543–76.

—— (1949). The embryology of *Caridina laevis* Heller. *Proc. Indian Acad. Sci.* **29**, 211–88.

NAIR, S. G. (1950). Two new species of *Irona* (Isopoda) parasitic on Madras fishes. *J. Madras Univ.* **20**, 66–74.

NICHOLLS, A. G. (1931). Studies on *Ligia oceanica. J. Mar. biol. Ass. U.K.* **17**, 655–73.

NUSBAUM, J. (1886). L'Embryologie d'*Oniscus murarius. Zool. Anz.* **9**, 454–8.

—— (1891*a*). Beiträge zur Embryologie der Isopoden. *Biol. Zbl.* **11**, 42–49.

—— (1891*b*). Zur Morphologie der Isopoden Füße. *Biol. Zbl.* **11**, 353–6.

—— (1898). Zur Entwicklungsgeschichte des Mesoderms bei den parasitischen Isopoden. *Biol. Zbl.* **18**, 557–69.

NUSBAUM-HILAROWICZ, J. (1917). Studien über die Physiologie der Verdauung bei den Landasselen (Isopoda). *Biol. Zbl.* **37**, 49–55.

RATHKE, H. (1834). Recherches sur la formation et le développement de l'Aselle d'eau douce (*Oniscus aquaticus* Linn.). *Ann. Sci. nat., Zool.* **11**, 139–57.

REINHARD, W. (1887). Zur Ontogenie des *Porcellio scaber. Zool. Anz.* **10**, 91–96.

ROULE, L. (1889). Sur l'évolution initiale des feuillets blastodermiques chez les Crustaces isopodes (*Asellus aquaticus* L. et *Porcellio scaber* Latr.). *C.R. Acad. Sci., Paris*, **109**, 78–79.

—— (1890). Sur le développement du blastoderme chez les Crustaces isopodes (*Porcellio scaber* Latr.). *C.R. Acad. Sci., Paris*, **110**, 1373.

Roule, L. (1891). Sur le développement des feuillets blastodermiques chez les Crustaces isopodes (*Porcellio scaber*). *C.R. Acad. Sci., Paris*, **112,** 100–41.

—— (1894). Études sur le développement des Crustaces. *Ann. Sci. nat., Zool.* sér. 7, **18,** 1–156.

—— (1896). Études sur le développement des Crustaces. La segmentation ovulaire et le façonnement du corps chez l'*Asellus aquaticus* L. *Ann. Sci. nat., Zool.* sér. 8, **1,** 163–96.

Sollaud, S. (1923). Recherches sur l'embryogénie des Crustaces décapodes de la sous-famille des Palaemoninae. *Bull. biol.*, suppl., **5,** 1–234.

Terao, A. (1929). On the embryonic development of the spiny Lobster *Panulirus japonicus* (v. Siebold). *Jap. J. Zool.* **2,** 387–449.

EXPLANATION OF FIGURES

Abbreviations

a.a., anterior aorta. *a.1*, antennule. *a.2*, antenna. *bl.*, blastomeres below the peripheral protoplasm. *bl.a.*, blastoporal area. *bl.d.*, blastodisc. *bls.*, blastomeres on the surface of the egg. *c.*, circle of ten blastomeres. *c.c.*, corrugated cuticle. *c.d.*, cavity inside the dorsal organ. *ch.*, chorion. *c.l.*, cephalic lobe. *cl.r.*, cephalic rudiment. *c.l.r.*, cephalic lobe rudiment. *c.m.b.*, central mesodermal block. *c.n.*, central nucleus. *c.p.*, central protoplasm. *coe.c.*, coelomic cavity. *c.r.*, crescentic row of blastoderm cells. *d.a.*, dorsal aorta. *d.c.*, degenerating cells. *d.m.*, dorsal muscles. *d.m.b.*, dorsal mesodermal block. *d.o.*, dorsal organ. *d.s.*, dorsal septa in the pericardial cavity. *e.*, eye. *end.*, endoderm. *end.a.*, area of endoderm formation. *e.t.*, ectodermal teloblast. *f.d.m.b.*, fork of the dorsal mesodermal block. *g.*, gut. *g.m.r.*, gastric mill rudiments. *h.*, heart. *i.g.*, inner group of segmental mesoderm cells. *l.m.*, limb mesoderm. *li.ce.*, liver lobe. *li.ru.*, liver rudiments. *lu.li.*, lumen of the liver tubes. *man.*, mandible. *man.p.*, mandibular palp. *max.1*, maxilla 1. *max.2*, maxilla 2. *max.p.*, maxilliped. *m.en.*, mesendodermal cells. *m.en.p.*, mesendodermal plug. *m.g.*, middle group of segmental mesoderm cells. *m.g.ist.*, mesodermal investment of the gut. *m.m.b.*, middle mesodermal block. *m.t.*, mesodermal teloblast. *n.g.*, nerve ganglion. *o.g.*, outer group of segmental mesoderm cells. *oes.*, oesophagus. *op.r.*, optic rudiment. *p.c.*, pericardial cavity. *pl.1–5*, pleopods 1–5. *p.m.*, pre-antennulary mesoderm. *p.m.a.*, anterior strand of the pre-antennulary mesoderm. *p.m.c.*, coelomic cavity of the pre-antennulary mesoderm. *p.m.ist.*, investment of the pre-antennulary mesoderm over stomach. *p.m.l.*, labral branch of the pre-antennulary mesoderm. *p.m.r.*, rudiment of the pre-antennulary mesoderm. *p.p.*, peripheral protoplasm. *pr.*, proctodoeum. *p.rid.*, posterior ridge of the dorsal organ. *p.w.*, pericardial wall. *st.*, stomodoeum. *st.f.*, flask-shaped stomodoeal sac. *sth.*, stomach. *tel.*, telson. *th.l.1–6*, thoracic legs 1–6. *t.m.*, telson mesoderm. *ur.*, uropod. *v.*, vacuole. *v.m.b.*, ventral mesodermal block. *vi.*, vitellophag cell. *v.s.*, ventral septa in the pericardial cavity. *y.*, yolk. *y.b.*, yolk fragment. *y.g.*, yolk globule. *y.m.*, yolk membrane. *y.p.*, yolk particle.

Descriptions of Figures

1. Section of the extruded egg.
2. Section of the egg at the eight-celled stage, before the blastomeres have emerged from the yolk. Two blastomeres are seen.
3. Lateral view of the egg at the eight-celled stage.
4. Lateral view of the egg at the sixteen-celled stage showing the ring of ten cells.
5. Lateral view of the egg at the thirty-two-celled stage, slightly tilted to show the ring of ten cells.
6. Lateral view of the egg showing migration of cells to form the blastodisc.
7. Blastodiscs, being completed, on surface of the egg. (Blastomeres on the opposite side are dark.)
8. Surface view of the blastodisc before gastrulation showing the crescentic row of cells and three proliferating areas.
9. L.S. of germinal disk of the same age as Fig. 8, before gastrulation.
10. L.S. of a blastodisc showing immigration of a single cell.
11. L.S. of a blastodisc showing a few cells immigrated.

12. L.S. of a blastodisc showing the shallow depression (the blastoporal homologue).
13. L.S. of the blastodisc showing the mesendodermal plug.
14. L.S. of the mesendodermal plug showing degenerating cells.
15. Nuclei of cells at different stages of degeneration:
   (*a*) nucleolus globular; two other globules have also appeared inside the nuclear reticulum;
   (*b*) globules increased in size;
   (*c*) two of the globules have fused;
   (*d*) nucleus before rupturing; a large globule has almost filled the entire nucleus.
16. Surface view of the blastodisc showing the ectodermal and mesodermal teloblasts.
17. Slightly sagittal section of the embryo of the same age as Fig. 16, showing ectodermal and mesodermal teloblasts.
18. Surface view of the blastodisc showing the teloblastic row straightened.
19. L.S. of the embryo of the same age as Fig. 18.

20–28. Post-naupliar stages as described on pp. 7–8.

29–33. Diagrammatic representation of the development of heart, liver, and gut at various stages of growth.

29–32. Lateral view of the embryos represented in Figs. 25–28.

33. Lateral view of the newly hatched larva.
34. L.S. of the embryo of the same age as in Fig. 21; the rudiments of all body segments are established.

35–37. Three stages in the formation of the stomodoeum.

38–39. Two stages in the development of the proctodoeum.

40. L.S. of the embryo of the same age as in Fig. 23, showing the cuticular lining receding from the invaginated sac (dorsal organ).
41. L.S. of an embryo of the same age as in Fig. 26, showing the cuticle becoming corrugated.
42. T.S. of an embryo of the same age as in Fig. 27, passing through the posterior ridge of the dorsal organ showing the two cavities; yolk particles seen inside are dark.
43. Parasagittal section of an embryo of the same stage as in Fig. 20, showing the immigration of the pre-antennulary mesodermal cells.
44. Parasagittal section of an embryo of the same age as in Fig. 21. Pre-antennulary mesoderm is growing backwards as a solid strand, from its ectodermal connexion.
45. T.S. of an embryo older than in Fig. 31, showing the course of the pre-antennulary mesodermal strands and the coelomic cavity fully developed.
46. T.S. of an embryo older than in Fig. 32, showing the formation of the dorsal aorta from the pre-antennulary mesoderm.

47–53. Sections showing the development of the liver.

47. T.S. through the maxillulary segment of an embryo of the same age as in Fig. 22, showing the liver rudiment lateral to the nerve ganglion.
48. Embryo as in Fig. 23. T.S. showing the curling of the liver rudiment dorsally.
49. Embryo as in Fig. 25. T.S. showing the liver rudiment forming a sac.
50. Embryo as in Fig. 26. T.S. showing the lobing of the liver sac.
51. T.S. through the second thoracic segment of an embryo showing the three liver lobes cut in a transverse plane; vacuoles are being formed in the liver cells.

52–53. T.S. and L.S. respectively of the liver lobes of the larva showing the absorption of the yolk by the liver cells. Yolk particles of varying sizes are represented as black dots.

54. T.S. through the posterior region of an embryo as in Fig. 21 showing the eight mesodermal teloblasts.
55. T.S. of the same embryo as in Fig. 54 passing through the second thoracic segment showing the three groups of mesoderm cells in the segmental region.
56. T.S. through the inter-segmental region between the second and third thoracic segments of a slightly older embryo than the last showing the single mesodermal strand.
57. Same embryo as in Fig. 23. Transverse section through the fourth abdominal segment showing the formation of the coelomic cavity in the dorsal mesodermal block.
58. Transverse section through the third abdominal segment of an embryo of the same age as in Fig. 26, showing the dorsal block of mesoderm forking to form the heart.

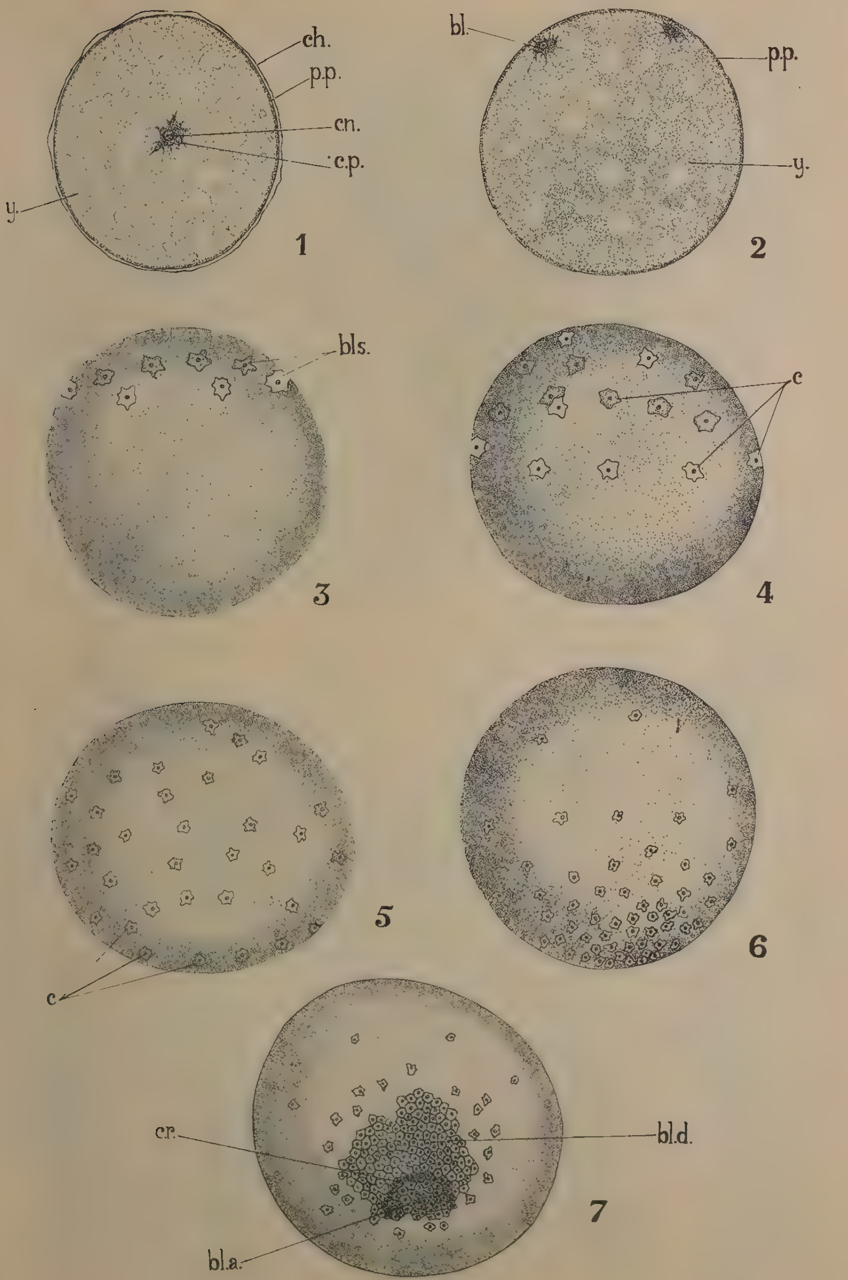
59. T.S. through the fourth abdominal segment of the larva showing the heart and the pericardium.

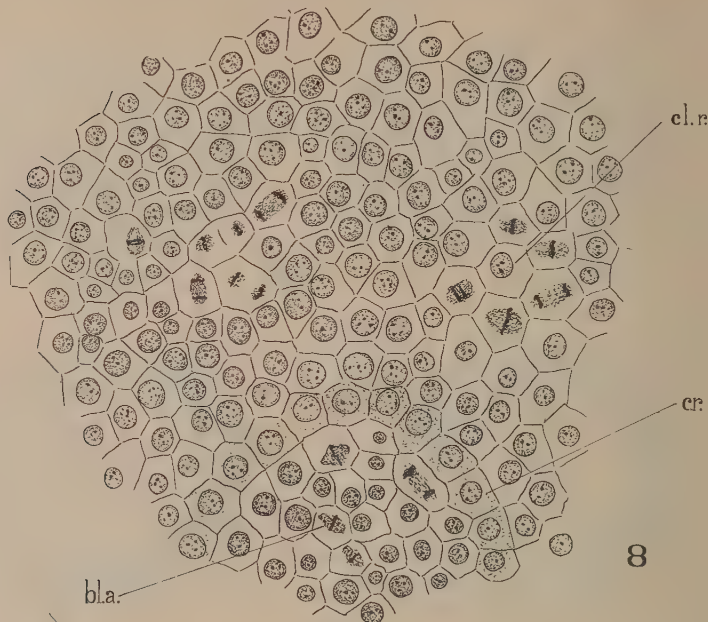
60. Lobing of the vitellophag nuclei before disintegration.

APPROXIMATE MAGNIFICATIONS

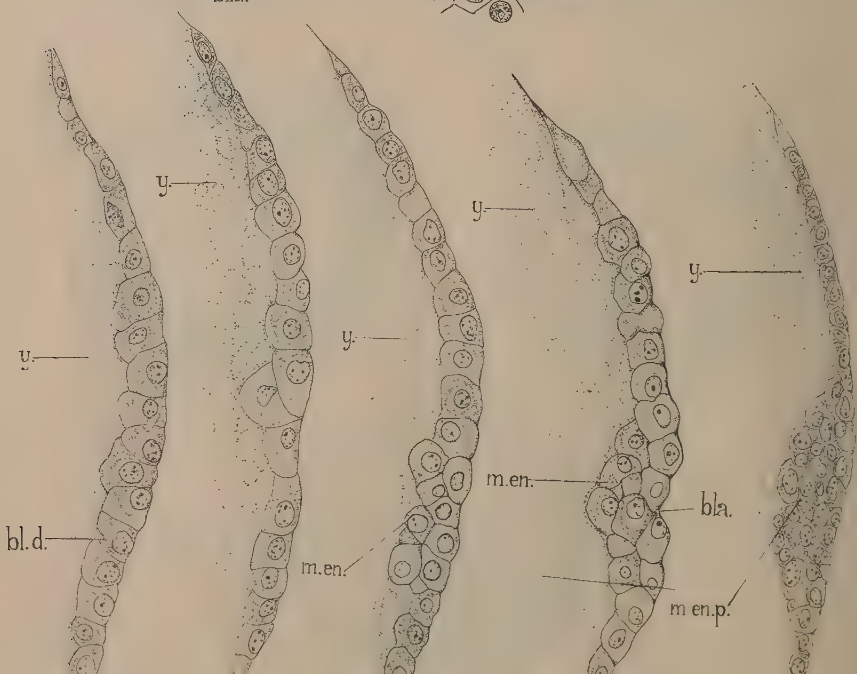
Figs. 1–7, ×32; 8–14, ×160; 15, ×360; 16–19, ×160; 20–33, ×32; 34–59, ×160; 60, ×360.

(Manuscript received 24:ii:55)

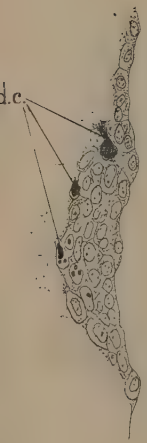

ch.
p.p.
cn.
c.p.
y.
1
bl.
p.p.
y.
2
bls.
3
c
4
c
5
6
cr.
bl.d.
bl.a.
7



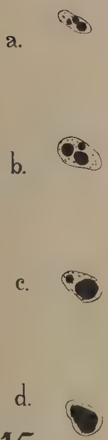
8



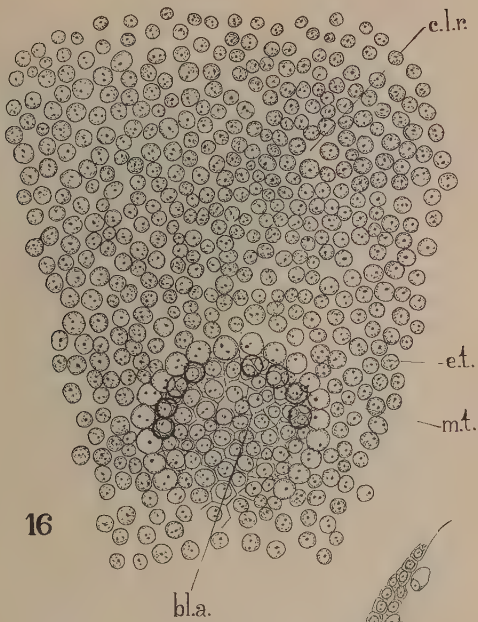
9 10 11 12 13



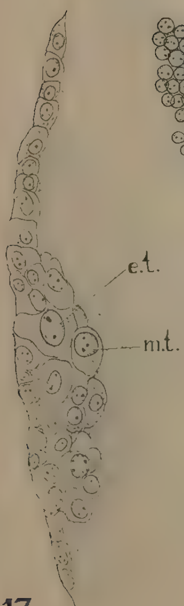
14



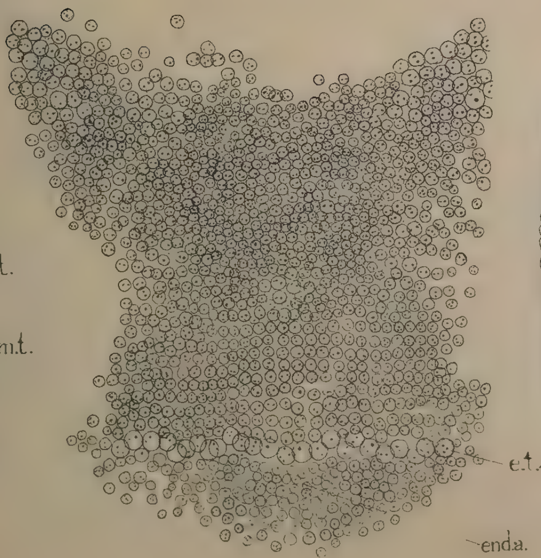
15



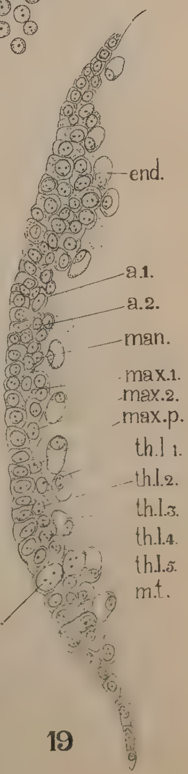
16



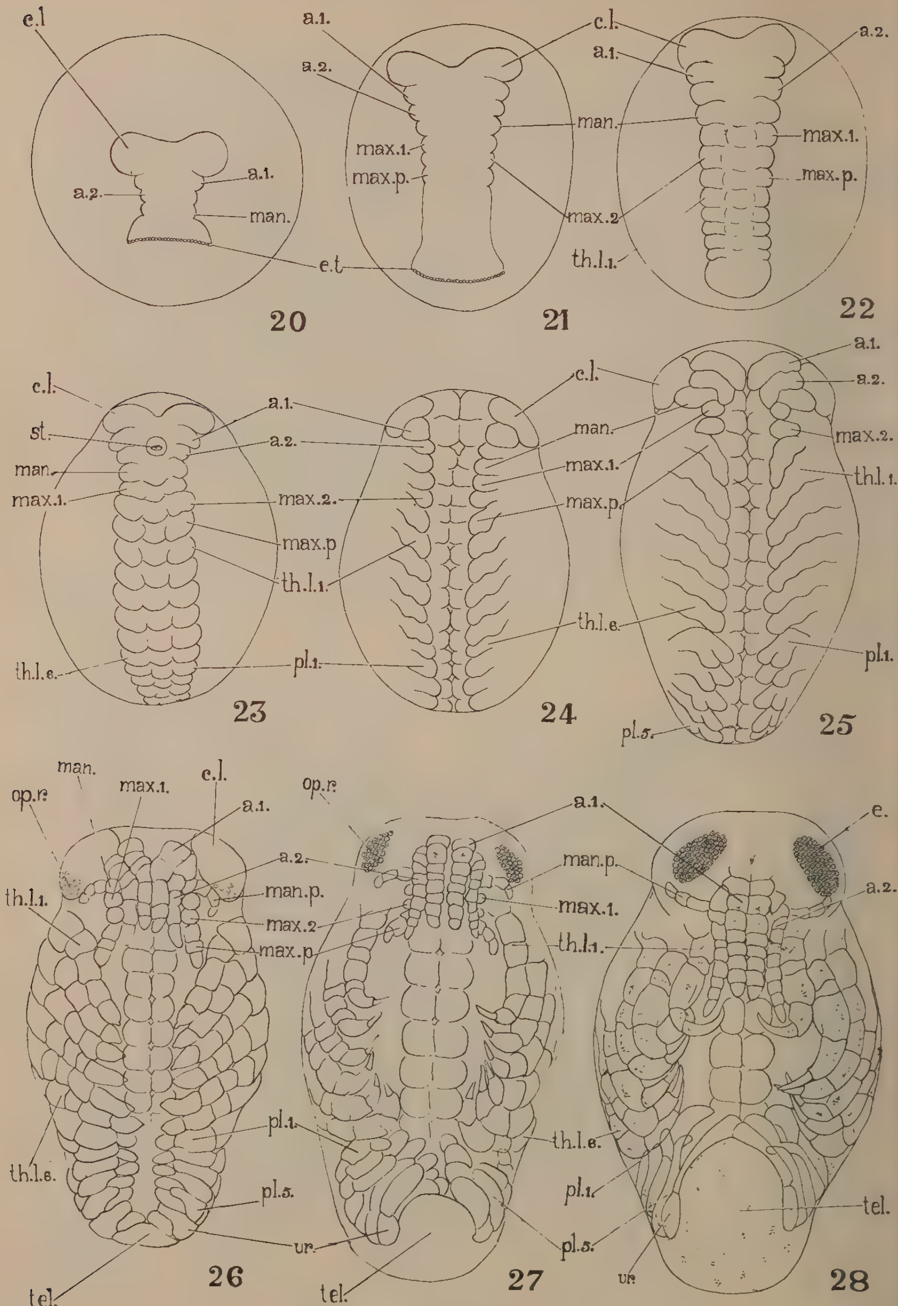
17

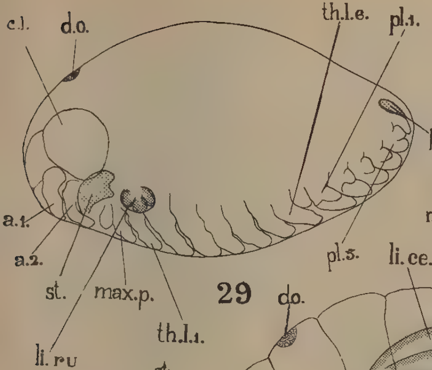


18

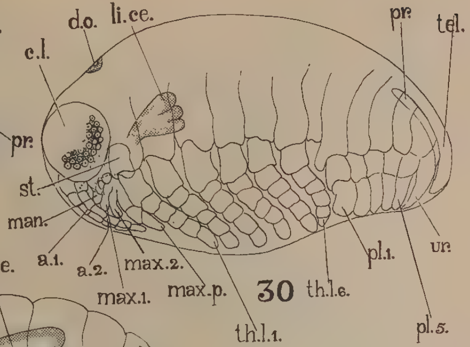


19

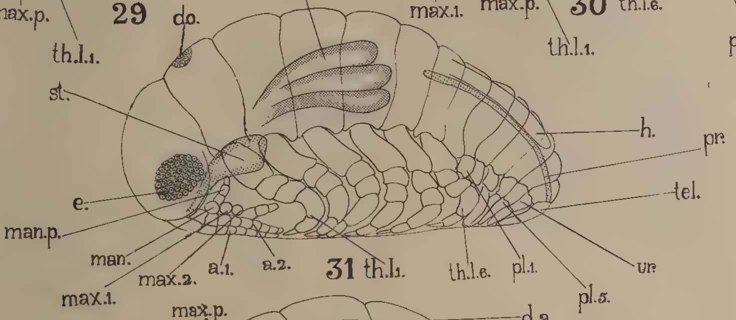

c.l
a.2.
a.1.
man.
e.t.
20
a.1.
a.2.
c.l.
max.1.
max.p.
man.
max.2
21
a.1.
a.2.
max.1.
max.p.
th.l.1.
22
c.l.
st.
man.
max.1.
a.1.
a.2.
max.2.
max.p
th.l.1.
th.l.6.
pl.1.
23
24
c.l.
man.
max.1.
max.p.
a.1.
a.2.
max.2.
th.l.1.
th.l.6.
pl.1.
pl.5.
25
man.
op.r.
max.1.
c.l.
a.1.
th.l.1.
a.2.
man.p.
max.2
max.p
pl.1.
th.l.6.
pl.5.
ur.
tel.
26
op.r.
a.1.
man.p.
max.1.
th.l.1.
th.l.6.
pl.1.
pl.5.
tel.
27
e.
a.2.
tel.
ur.
28


c.l.
d.o.
th.l.6.
pl.1.
pr.
a.1.
a.2.
st.
max.p.
li.ru
th.l.1.
pl.5.

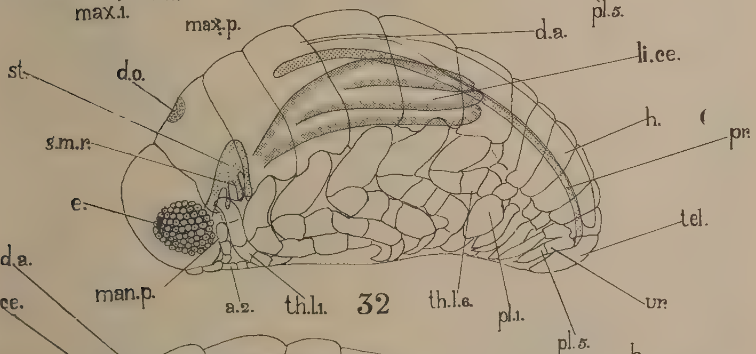
29


d.o.
li.ce.
c.l.
pr.
tel.
st.
man.
a.1.
a.2.
max.2.
max.1.
max.p.
th.l.1.
th.l.6.
pl.1.
ur.
pl.5.

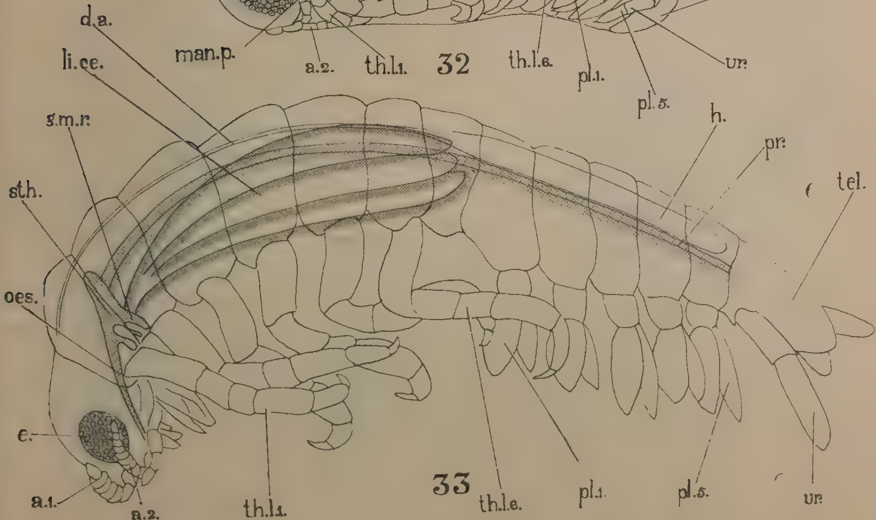
30


d.o.
li.ce.
st.
h.
pr.
e.
tel.
man.p.
man.
max.2.
max.1.
a.1.
a.2.
th.l.1.
th.l.6.
pl.1.
pl.5.
ur.
max.p.

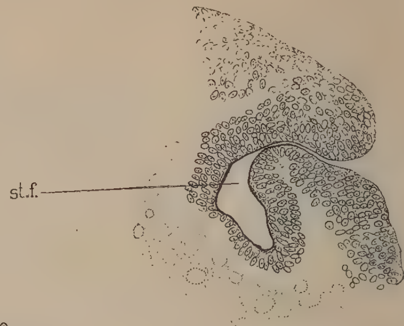
31


d.a.
li.ce.
st.
d.o.
h.
s.m.r.
pr.
e.
tel.
man.p.
a.2.
th.l.1.
th.l.6.
pl.1.
pl.5.
ur.

32

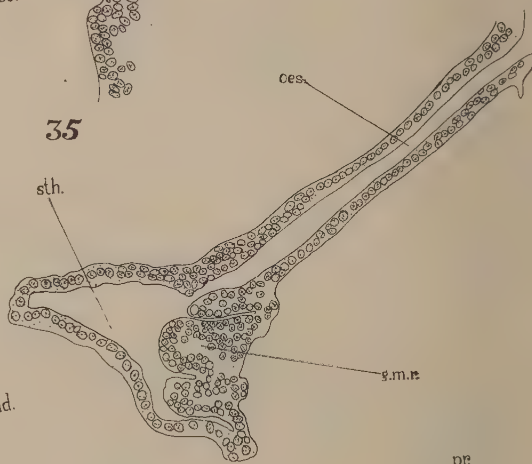

d.a.
li.ce.
s.m.r.
sth.
oes.
e.
a.1.
a.2.
th.l.1.
th.l.6.
pl.1.
pl.5.
h.
pr.
tel.
ur.

33



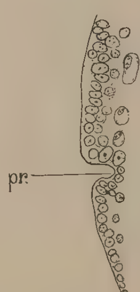
36

35

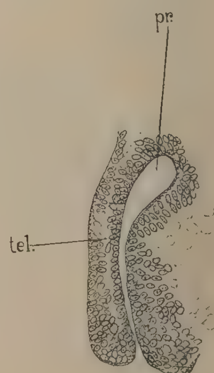


37

34



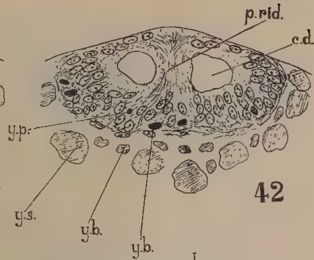
38



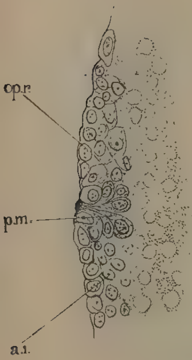
39

40

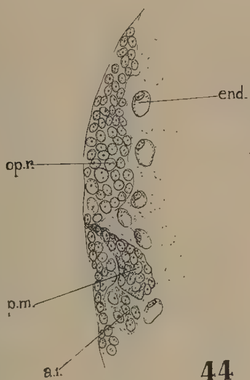
41



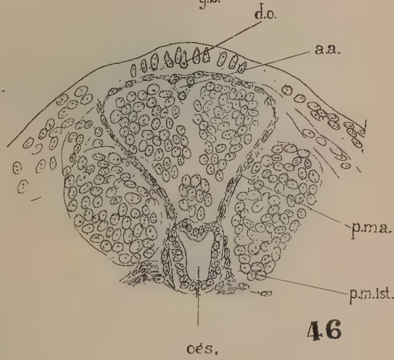
42



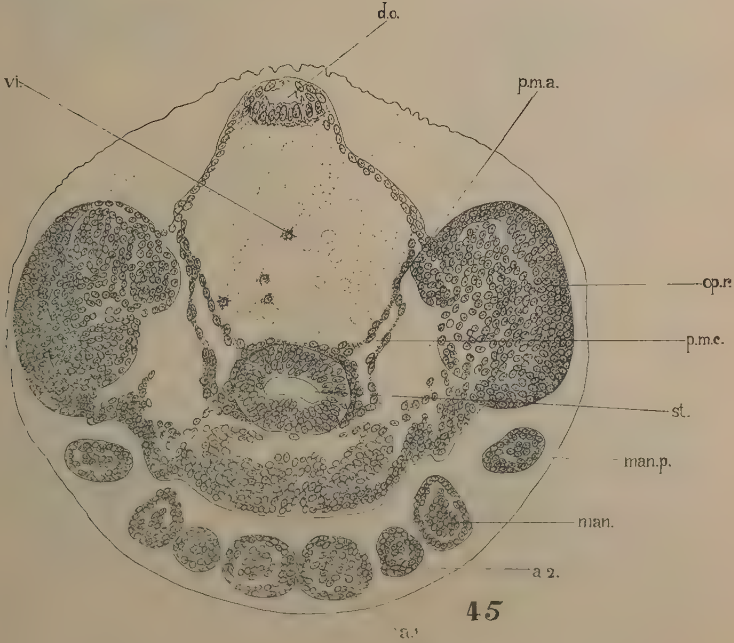
43



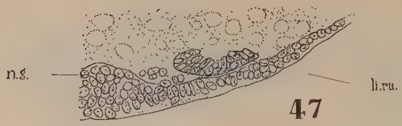
44



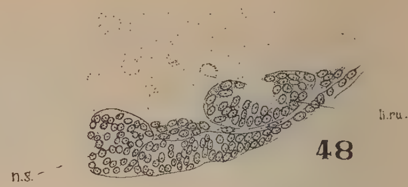
46



45

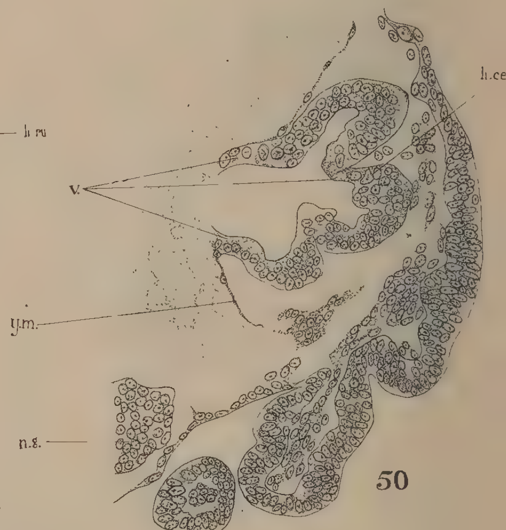


47

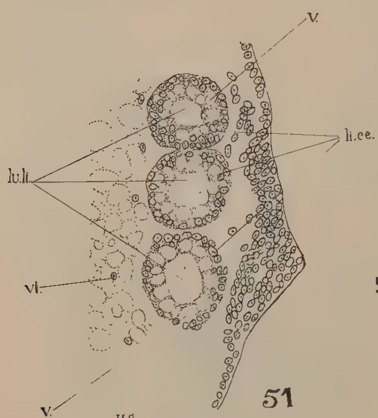


48

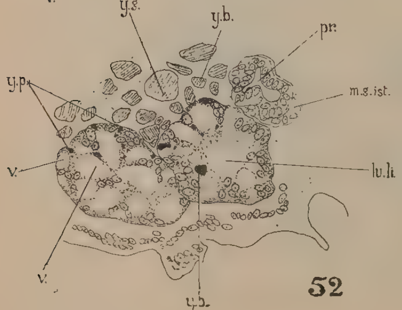
49



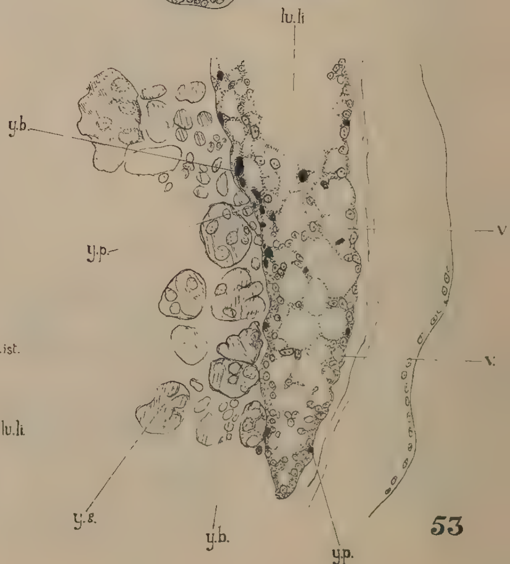
50



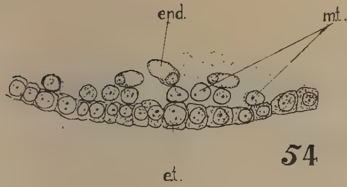
51



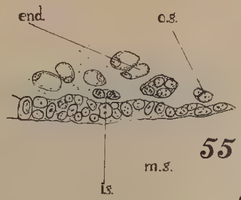
52



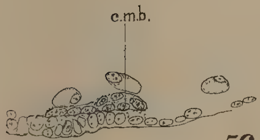
53



54



55



56

57

58

60

59

The Selective Transport of Antibodies from the Yolk Sac to the Circulation of the Chick

by JOAN BRIERLEY *and* W. A. HEMMINGS[1]

Agricultural Research Council's Unit of Embryology, University College of North Wales, Bangor

INTRODUCTION

It has been shown that in the rabbit the transfer of passive immunity from mother to foetus takes place through the yolk-sac wall (Brambell, Hemmings, Henderson, Parry, & Rowlands, 1949). This membrane is selective; when exposed experimentally to a mixture of antibodies produced in rabbit and other species, higher relative concentrations of rabbit antibody are found in the foetal circulation (Brambell, Hemmings, Henderson, & Rowlands, 1950). The heterologous antibodies enter at lower relative concentrations characteristic of each species. Thus man, guinea-pig, dog, horse, and cow antitoxins tested in this way, form a series in descending order (Batty, Brambell, Hemmings, & Oakley, 1954). It is of interest to determine if other membranes through which maternal immunity is transmitted to the young display a comparable selectivity. The yolk-sac of the chick provides a suitable and convenient membrane to examine, since passive immunity is known to be transmitted from mother to young by way of the yolk of the egg (Klemperer, 1893; Brandly, Moses, & Jungherr, 1946; Buxton, 1952). The researches described herein were intended to explore this problem.

TECHNIQUE

Injection into the yolk of the egg was made by the method described by Beveridge & Burnet (1946). A hole was drilled over the air space, and the needle inserted to a depth of 3 cm. Injection into the yolk-sac of the newly-hatched chick was made through skin and body wall, which was rendered translucent by alcohol swabbing. This procedure is no more severe than subcutaneous injection. It involves negligible hazard to the chick (in contrast with the first method, where considerable mortality was encountered), is certain, and technically simple. For these reasons it was preferred for the bulk of the experiments.

To control the risk of leakage into the peritoneum, Evan's blue was added to the injection sera to a concentration of 1 mg./ml. The dye was never seen in the circulation, but the splanchnopleur was invariably heavily stained.

[1] *Authors' address*: Department of Zoology, University College of North Wales, Bangor, U.K.

[J. Embryol. exp. Morph. Vol. 4, Part 1, pp. 34–41, March 1956]

All experiments were conducted under aseptic conditions: sera were sterilized by centrifugal filtration. At the end of an experiment the chicks were bled out from the right jugular under ether anaesthesia.

Yolk was extracted by the method of Buxton (1952) with 4 volumes of 8 per cent. saline, the aqueous layer being used as a 1/5 dilution for titration. The possibility that this method might alter the demonstrable titre of sera added to the yolk was tested, but no change was found. Doubling dilutions of serum were set up against standard antigen; anti-Brucella reactions were read at a + end-point, anti-Salmonella at + +.

To present results in comparable form, experimental titres are expressed as a fraction of the titres of the sera injected. Over the range of titres met in this work the error so introduced is negligible. The fraction is termed the concentration quotient (c.q.). Sera were injected as available, without selection for uniformity of titre, and in most experiments more than one serum was employed. The number of experiments available at the lower c.q. values therefore declines, and for this reason results are expressed as the percentage of reactions positive at each dilution.

RESULTS

Occurrence of antibodies in the egg-yolk

Antibodies occur in the yolks of eggs laid by immune birds. The egg-yolk and serum titres of four laying hens that had been actively immunized to *Salmonella pullorum* are given in Table 1. Ideally the yolk-titre should be compared with that of the serum at the time when the yolk was being secreted which according to Lillie (1927) is 5 to 8 days before laying. In practice test bleedings tended to

TABLE 1

Relation of serum and egg-yolk titres of immune laying hens

The figures indicate the number of eggs tested

Titre of antibody in fowl serum	1/10	1/20	1/40	1/80	1/160	1/320
1/2560					1	2
1/1280			1		1	
1/640	2		4	5		
1/320			1			
1/160		11	1			
1/80	2					

Titre of antibody in egg-yolk

put the birds off laying and for this reason the yolk-titre was compared with that of the serum at the last bleeding preceding laying, the interval varying from 1 to 17 days. It is apparent that a close relation exists between the antibody titres in the serum and in the yolk, the c.q. of the yolk approximating to 1/8.

The antibody titre of the yolk of a fertile egg does not appear to fall during incubation. Five chicks ranging from 1 to 4 days old, hatched from eggs laid by

the hens referred to above, had residual titres of 1/80 to 1/320 to *S. pullorum* in the small volume of yolk remaining. Similarly, antibodies injected into the yolk-sac on the 6th day of incubation, were present in the yolk-sac of the newly-hatched chick.

An attempt was made to find if there was any significant decrease of titre in yolk incubated *in vitro* due to proteolysis. Immune sera were mixed with yolk, withdrawn either from freshly laid eggs or from developing eggs on the 6th day of incubation, and were incubated under sterile conditions at 39° C. No fall in titre was observed after 13 days.

Injection of immune fowl serum into the yolk-sac

Immune fowl serum was injected into the yolk-sac on the 6th or 8th day of incubation. The dose administered was 0·5 to 1·0 ml. of antiserum to *Brucella abortus* of agglutinin titres between 1/640 and 1/2560. Thirty-five eggs hatched in this experiment and the chicks were killed within 5 days, the sera being

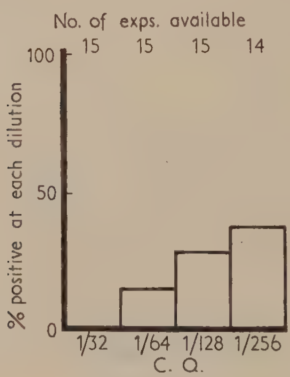


FIG. 1

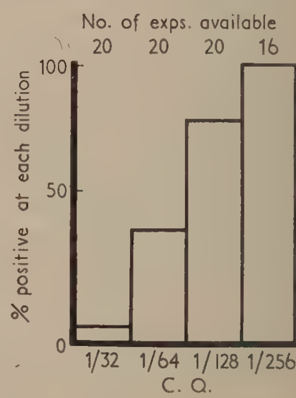


FIG. 2

TEXT-FIG. 1. Circulating antibody concentration quotient of chicks following injection of immune fowl serum into the yolk at 6 days.

TEXT-FIG. 2. Circulating antibody concentration quotient of chicks following injection of immune fowl serum into the yolk at 8 days.

titrated. The results are summarized in Text-figs. 1 and 2; clearly antibody was taken up in substantial amount. It is hard to account for the significantly slighter uptake following injection at 6 days, unless it be due to the greater turgidity of the yolk at this stage causing leakage of serum.

Immune fowl serum was injected into the retracted yolk-sac of 61 newly-hatched chicks. Injections performed up to 48 hours after hatching resulted in the appearance of antibody in the circulation. The chicks were killed within 7 days of hatching, the majority 1 to 4 days after hatching. An interval of at least

24 hours elapsed in all cases between injection and killing. Brandly, Moses, & Jungherr (1946) have shown that the serum antibody titres of chicks of immunized hens remain at peak levels during two weeks after hatching. Hence, the age within these limits at which the chick serum is harvested is unimportant. Antiserum to *Salmonella pullorum* was administered to 38 chicks, and antiserum to *Brucella abortus* to 23 chicks. The antiserum to *Salmonella pullorum* had an agglutinin titre of 1/640 to 1/1280 and the dose varied from 0·5 to 1·0 ml. The antiserum to *Brucella abortus* had an agglutinin titre of 1/1280 to 1/2560 and the dose was 0·7 to 0·8 ml. The results are summarized in Text-fig. 3.

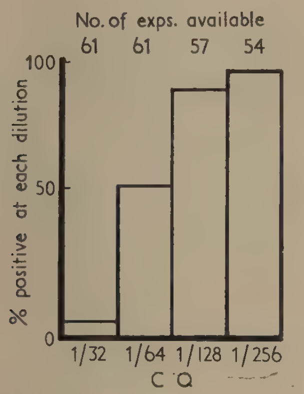


FIG. 3

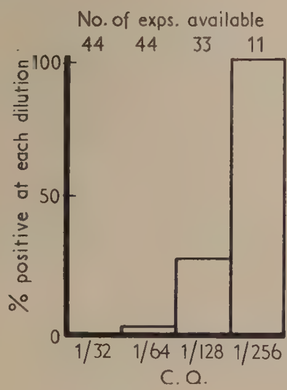


FIG. 4

TEXT-FIG. 3. Circulating antibody concentration quotient of chicks following injection of immune fowl serum into the yolk-sac of the newly-hatched.

TEXT-FIG. 4. Circulating antibody concentration quotient of chicks following injection of immune pigeon serum into the yolk-sac of the newly-hatched.

Injection of immune sera prepared in other species into the yolk-sac

Bovine antiserum to *Brucella abortus* with a titre of 1/1280 was injected into the yolk-sac on the 6th day of incubation, the dose being 1 ml. Five chicks hatching from these eggs had sera that were negative at a dilution of 1/10. The yolk remaining in the yolk-sacs of these chicks had titres of 1/80 to 1/640, which are of the order expected immediately after injection, assuming that the yolk-volume was *c.* 20 ml. It was decided therefore to employ the more sensitive test for antitoxins. Accordingly 1 ml. of bovine or equine anti-diphtheria serum was injected into the yolk-sac on the 6th day of incubation, the sera of the chicks being harvested during the first 4 days after hatching. Bovine antitoxin was absent from the sera of 5 chicks tested at c.q. 0·0002, but equine antitoxin was present in one of the two chicks tested at c.q. 0·00004.

Antisera to *Brucella abortus*, prepared in pigeon, rabbit, or cow, were injected into the yolk-sacs of chicks within 24 hours of hatching. The chicks were killed

within 6 days of hatching and not less than 24 hours after injection. The pigeon sera had titres of 1/640 to 1/2560 and the doses varied from 0·6 to 1·0 ml. The results are presented in Text-fig. 4. The rabbit sera had titres of 1/640 to 1/2560 and the dose was 0·6 or 0·7 ml. Rabbit antibody was absent from the sera of 10 chicks tested at c.q. 1/128, and of 5 at 1/256. The bovine sera had titres of 1/1280 or 1/2560 and the dose was 0·6 to 0·8 ml. Bovine antibody was absent from the sera of 10 chicks tested at c.q. 1/128, and of 5 at 1/256. These three series of experiments are comparable with each other. It is clear that only the pigeon antibody was present in the circulation and this at concentrations much lower than was the case with fowl antibody.

Oral administration of immune fowl serum to the newly-hatched chick

Fowl anti-*Brucella* serum was fed via a polythene stomach tube to 41 chicks ranging in age from hatching to 48 hours old. The dose administered was either 0·5 or 1·0 ml. of serum positive at 1/640 or 1/1280. Chicks were killed after an interval of from 4½ to 48 hours. The serum was negative in all cases. Nor was any residual titre found in the stomach and intestinal contents after 4½ hours. There was thus no uptake from the gut at a c.q. of 1/128.

DISCUSSION

The transmission of antibodies from the hen to the yolk of the egg was reported by Klemperer (1893), using birds actively immunized to tetanus toxin. Since then this observation has been confirmed many times. The results reported herein show that the agglutinin titre of the yolk is directly related to that of the maternal serum, being approximately 1/8 of the serum titre. Knight & Schechtman (1954) have reported that heterologous serum protein administered intravenously to the hen appears unaltered in eggs laid from 2 to 3 days thereafter. It is not precipitable with the livetin. The antibody activity in the egg is associated with the water soluble or livetin fraction of the yolk proteins (Jukes, Fraser, & Orr, 1934). Immunologically livetin is indistinguishable from serum globulin (Jukes & Kay, 1932). It has been shown (Shepard & Hottle, 1949) to be separable electrophoretically into three components, like serum globulin. Nace (1953), using specific rabbit precipitating antisera, found that globulin appears in the chick circulation about the 12th day of incubation, considerably later than the other major components. Marshall & Deutsch (1950) found that all the adult electrophoretic peaks are represented on the 10th day, but the relative proportions change as development proceeds: in particular there is a three- or four-fold increase in globulin during the few days prior to hatching. Antibody determinations are in agreement: thus Brandly, Moses, & Jungherr (1946) state that antibodies to Newcastle disease virus are not detectable in the circulation until the 18th day, and Buxton (1952) found that agglutinins to *Salmonella pullorum*

appear only at the 17th day, though a positive Coomb's test is given from the 11th. Thus although the full complement of antibody of the chick is present in the yolk at laying, it does not appear in the circulation until late in incubation.

The results reported here show that antibody continues to be absorbed from the yolk-sac after hatching. Comparison of the results of injection of immune serum into the yolk-sac at the 8th day and in the newly-hatched chick (Text-figs. 2 and 3) shows that the latter method gives circulating concentrations at least as high as the former. In both cases, however, the circulating antibody at killing accounts for no more than a few per cent. of that injected, under the conditions of dosage adopted in this work. The antibody concentration in the yolk does not seem to be reduced during incubation. Although proteolytic enzymes are demonstrable in yolk (Remotti, 1927; Goldstein & Gintsbourg, 1936) they do not lower the antibody titre significantly when immune serum is incubated *in vitro* with yolk for long periods. Thus the fate of the larger part of the antibody injected into the yolk in these experiments, and by implication that of the native livetin, is still uncertain.

The experiments with immune pigeon serum injected into the yolk-sac of the newly-hatched chick show that antibodies prepared in the pigeon can pass into the circulation. Comparison of Text-figs. 3 and 4 show that the pigeon antibodies were present in the circulation of the chick at lower concentrations than fowl antibodies. Antibodies from none of the mammalian sera employed, whether agglutinins or antitoxins, entered the chick circulation in significant amounts. Thus the yolk-sac of the chick, like that of the rabbit, is selective; and a mechanism must be assumed which permits, but closely regulates, entry of unaltered protein to the circulation.

The yolk-sac is connected to the gut of the chick by the yolk-sac stalk. Although the lumen of the yolk-sac stalk appears to be patent, yolk does not pass through it into the intestine, according to Lillie (1927). Oral administration of immune serum to the newly-hatched chick shows that antibody is not absorbed from the gut. Hence it is evident that the absorption of antibody from the yolk must occur via the yolk-sac. The fowl is similar to the rabbit, therefore, in that the transfer of passive immunity from the mother occurs through the yolk-sac splanchnopleur into the vitelline circulation of the area vasculosa. In the chick, owing to the retraction and consequent retention of the yolk-sac, absorption continues and is, indeed, most effective after hatching. In the rabbit, the transfer of passive immunity is pre-natal: the splanchnopleur is lost at parturition. Neither the chick nor the rabbit appears to absorb antibody from the gut and in this they differ from the ruminants, which acquire their passive immunity from the colostrum or milk by way of the gut of the newborn.

SUMMARY

1. The yolk titre of eggs laid by hens actively immune to *Salmonella pullorum* is directly related to the serum titre, the concentration quotient being approximately 1/8.

2. Fowl sera immune to *S. pullorum* or *Brucella abortus* when injected into the yolk-sac early in incubation give rise to circulating titres in the chick at hatching.

3. A technique of injection into the yolk-sac after hatching is described. Immune fowl serum so injected gives rise to circulating titres.

4. Immune rabbit, cow, and horse sera so injected do not give rise to circulating titres. Immune pigeon serum gives rise to titres at lower concentration quotient than fowl. It is concluded that the yolk-sac splanchnopleur is selective.

5. Fowl antibody fed by mouth after hatching does not give rise to circulating titres.

ACKNOWLEDGEMENTS

We wish to express thanks to Professor F. W. R. Brambell, F.R.S., for his interest and advice; and to Dr. C. L. Oakley for undertaking antitoxin determinations. We are indebted to Dr. A. W. Stableforth of the M.A.F. Veterinary Laboratories, Weybridge, and Mr. W. T. Rowlands, M.A.F. Veterinary Advisory Service, for supplies of immune sera and standard antigens.

REFERENCES

Batty, I., Brambell, F. W. R., Hemmings, W. A., & Oakley, C. L. (1954). Selection of antitoxins by the foetal membranes of rabbits. *Proc. roy. Soc.* B, **142,** 452–71.

Beveridge, W. I. B., & Burnet, F. M. (1946). *The Cultivation of Viruses and Rickettsiae in the Chick Embryo.* Med. Res. Coun., London, Special Rep. No. 256.

Brambell, F. W. R., Hemmings, W. A., Henderson, M., Parry, H. J., & Rowlands, W. T. (1949). The route of antibodies passing from the maternal to the foetal circulation in rabbits. *Proc. roy. Soc.* B, **136,** 131–44.

—— —— —— & Rowlands, W. T. (1950). The selective admission of antibodies to the foetus by the yolk-sac splanchnopleur in rabbits. *Proc. roy. Soc.* B, **137,** 239–52.

Brandly, C. A., Moses, H. E., & Jungherr, E. L. (1946). The transmission of antiviral activity via the egg and the role of congenital passive immunity to Newcastle disease in chickens. *Amer. J. vet. Res.* **7,** 333–42.

Buxton, A. (1952). On the transference of bacterial antibodies from the hen to the chick. *J. gen. Microbiol.* **7,** 268–86.

Goldstein, B., & Gintsbourg, M. (1936). Proteinases (cathepsin) in the tissues of the chicken embryo. *Enzymologia,* **1,** 369–72.

Jukes, T. H., Fraser, D. T., & Orr, M. D. (1934). The transmission of diphtheria antitoxin from hen to egg. *J. Immunol.* **26,** 353–60.

—— & Kay, H. D. (1932). The immunological behaviour of the second protein (livetin) of hen's egg yolk. *J. exp. Med.* **56,** 469–82.

Klemperer, F. (1893). Über natürliche Immunität und ihre Verwerthung für die Immunisirungstherapie. *Arch. exp. Path. Pharmak.* **31,** 356–82.

KNIGHT, PATRICIA F., & SCHECHTMAN, A. M. (1954). The passage of heterologous serum protein from the circulation into the ovum of the fowl. *J. exp. Zool.* **127,** 271–304.

LILLIE, F. R. (1927). *The Development of the Chick.* New York: Henry Holt and Co.

MARSHALL, M. E., & DEUTSCH, H. F. (1950). Some protein changes in fluids of the developing chicken embryo. *J. biol. Chem.* **185,** 155–61.

NACE, G. W. (1953). Serological studies of the blood of the developing chick embryo. *J. exp. Zool.* **122,** 423–48.

NEEDHAM, J. (1942). *Biochemistry and Morphogenesis.* Cambridge: The University Press.

REMOTTI (1927). Quoted by Needham.

SHEPARD, C. S., & HOTTLE, G. A. (1949). Studies of the composition of the livetin fraction of the yolk of hen's eggs with the use of electrophoretic analysis. *J. biol. Chem.* **179,** 349–57.

(Manuscript received 2:iv:55)

The Time and Place of Fertilization of the Golden Hamster Egg

by FRITZ STRAUSS[1]

From the Departments of Anatomy, University of Wisconsin, Madison, Wisconsin, and University of Berne, Switzerland

INTRODUCTION

THERE is little precise information in the literature as to the exact place where the union of the male and female gametes occurs in the genital tract of mammals. Yet it seems that an exact knowledge of the place of fertilization might be of great significance, not only for our understanding of the process of fertilization itself, but in its relation to the manifold problems associated with the limited span of time during which the capacity for fertilization persists in the sperm and egg, and to the physiology of later development and implantation.

In most writings about the early development of Man and other mammals, it is assumed that the egg is fertilized in the cephalic part of the oviduct. However, there is still insufficient evidence to justify such general assumptions. It would not be surprising if it should be shown that different species have different topographical positions of the fertilization site correlated for instance with such factors as spontaneous versus induced ovulation.

Such an unsatisfactory situation suggests the necessity of a review of our knowledge concerning the site of fertilization of the mammalian egg, and motivated me to investigate this problem.

MATERIAL AND METHODS

This first study is based on the examination of 780 fertilized and unfertilized eggs from 66 golden hamsters, *Mesocricetus auratus* Waterhouse. The hamster was chosen because of its exact adherence to an easily determined sexual cycle. It ovulates spontaneously every 4 days in the early morning hours between 1 and 4 o'clock of the second day of the estrous cycle (Ward, 1946; Boyer, 1953). The ova are discharged at any stage from 'first anaphase' to 'first polar body, second metaphase' (Ward, 1948*a*). The animals used in this investigation were selected on the basis of simple daily vaginal inspection using the data of Ward (1946). This method has proved to be without failure.

[1] *Author's address*: Department of Anatomy, University of Berne, 26 Buehlstrasse, Berne, Switzerland.

[J. Embryol. exp. Morph. Vol. 4, Part 1, pp. 42–56, March 1956]

Instead of the copulation and ovulation age of most authors (Graves, 1945; Venable, 1946; Boyer, 1953), I started my experiments using the developmental age of the fertilized ova as defined by Ward (1948*b*) but subsequently changed to a more realistic time scale, based on a revised estimate of ovulation time, as a result of observations to be given later. Developmental age is the time span from the earliest possible fertilization time of an egg until its immersion in fixative. The developmental ages included in my series were 2, 3, 4, 5, 6, 12, 18, 24, and 30 hours according to Ward's data on ovulation time. For each age half of the animals had short periods for copulation in the evening (8.00–8.30 p.m.) *before* ovulation. The other half had a half-hour period for copulation in the morning about 7 a.m. *after* ovulation. Since Ward assumes the time of ovulation is 1 a.m., the developmental age and ovulation age would correspond in animals mated in the evening; in the animals mated in the morning the developmental age would be 6 hours less than the ovulation age. Unmated animals killed at the same time as these experimental series formed controls.

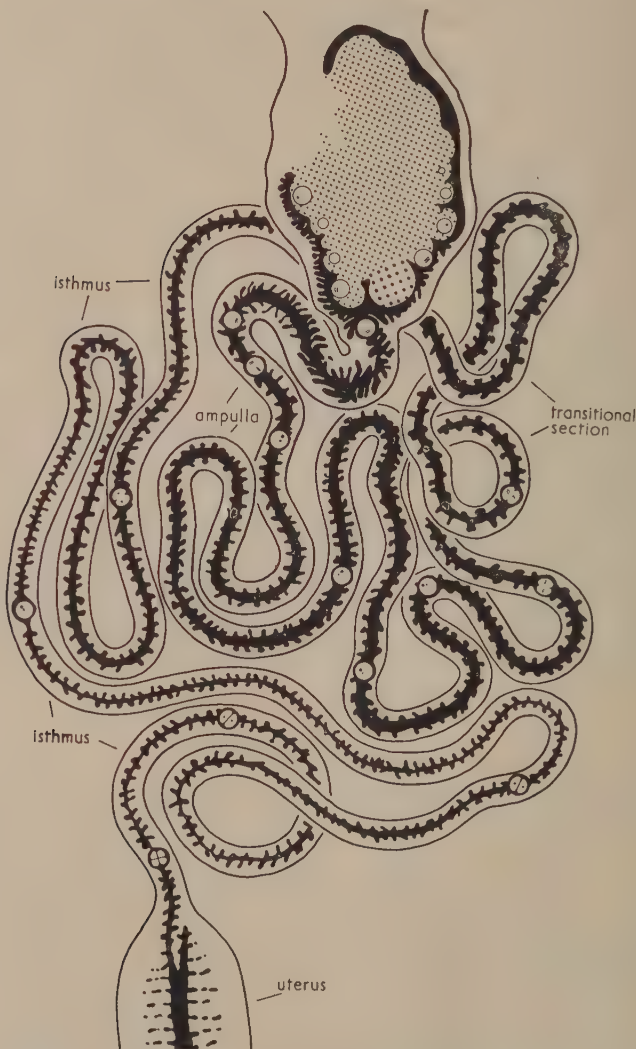
The entire genital organs were fixed immediately after death in Bouin's fluid. The ovaries with the oviducts and connecting parts were embedded in paraffin from cyclohexanon, sectioned at 8 μ, and stained with iron hematoxylin (Weigert) without counterstain.

The tortuous pattern of the uterine tubes of the golden hamster made it extraordinarily difficult to establish the exact position of the tubal eggs. Therefore it was necessary to draw the whole oviduct section by section after cutting, recording the relative position of the female gametes or zygotes. At the same time histological differences in the construction of the wall of various levels of the oviduct were noted.

OBSERVATIONS

The hamster ovary is, as in many rodents, completely enclosed by an ovarial sac which is continuous with the infundibulum of the oviduct (Text-fig. 1). The length of the infundibulum is about 0·2 mm. The transition from the smooth bursa wall to the funnel is characterized by the appearance of relatively high and slender mucous folds or fimbriae which contain only a very delicate connective tissue framework. The infundibulum leads into the ampulla, which is about 1·2 mm. long. Its wall is thin, relatively non-muscular, and contains low, wide-based mucosal ridges at rather regular intervals. Toward the isthmus the ampulla wall gradually increases in thickness, so that a sharp boundary between these two portions does not exist. It proved useful to designate a transitional section, about 0·6 mm. in length, between the ampulla and isthmus. The isthmus (approximately 1·7 mm. long) has a markedly narrower lumen than the preceding section of the oviduct, and carries numerous thick epithelial ridges which when cut perpendicularly appear somewhat club-shaped. In the transition from the isthmus to the uterine horn the number of ridges decreases, and they become

lower. The lumen here becomes indented by small separate protrusions. In the tunica propria glands appear; these increase in number and dimension and mark



TEXT-FIG. 1. Schematic drawing of the oviduct and ovary in *Mesocricetus auratus* Waterhouse. The various segments of the oviduct are differentiated by the different contours of the lumen.

the beginning of the uterus. In addition, a valve occurs at the tubal-uterine junction. In brief the oviduct of the hamster corresponds in structure and histology with that of the mouse as given by Sobotta (1895).

TABLE 1

Distribution and condition of the eggs from the mated animals according to hypothetical ovulation age (ovulation assumed at 3 a.m.) and time of mating

Mating m = morning e = evening	*Estimated ovulation age in hours*	*No. of animals*	*No. of corpora lutea*	*Total no. of ova*	*Mature ova within the ovary*	*No. of ovulated ova*	*No. of non-fertilized ova*	*No. of fertilized ova*	*No. of missing ova*
e . .	0	2	7	27	20	7	7	0	0
m . .	6	2	17	17	0	17	17	0	0
e . .	1	1	12	12	0	12	11	0	1
e . .	2	3	33	35	0	35	30	5	0
m . .	8	3	32	32	0	32	24	7	1
e . .	3	1	10	10	0	10	4	6	0
e . .	4	3	43	44	0	44	5	39	0
m . .	10	2	24	24	0	24	4	19	1
m . .	12	1	12	12	0	12	0	12	0
e . .	10	3	39	40	0	40	0	40	0
m . .	16	3	35	35	0	35	1	34	0
e . .	16	2	25	25	0	25	0	23	2
m . .	22	2	23	23	0	23	4	19	0
e . .	22	2	28	28	0	28	5	21	2
m . .	28	2	30	30	0	30	5	25	0
m . .	30	1	13	14	0	14	0	14	0
e . .	28	2	23	23	0	23	4	19	0
m . .	34	2	24	24	0	24	1	23	0
m . .	36	1	12	12	0	12	2	10	0

In Tables 1 (experimentals, mated) and 2 (controls, unmated) the distribution of 780 eggs in relation to their assumed ovulation age is presented. The eggs classed as 'missing' include both those for which a safe evaluation of their condition was not possible, and eggs which could not be found but which must have existed since there were more ruptured follicles or corpora lutea than recovered eggs.

TABLE 2

Distribution of the ova in the control (unmated) animals, grouped according to ovulation age to correspond with the experimental (mated) animals

Estimated ovulation age	*No. of animals*	*No. of corpora lutea*	*Total no. of ova*	*Mature ova within the ovary*	*No. of tubal ova*	*No. of missing ova*
0	2	7	22	14	7	1
6	2	21	21	0	19	2
2	2	23	23	0	23	0
8	2	17	17	0	17	0
4	2	24	24	0	23	1
10	2	24	24	0	23	1
10	2	24	25	0	24	1
16	2	20	20	0	20	0
16	2	27	27	0	27	0
22	2	22	22	0	22	0
22	2	20	20	0	18	2
28	2	25	25	0	25	0
28	2	20	20	0	19	1
34	2	23	23	0	22	1

Tables 1 and 2 permit an estimate of the number of ova which must have been produced from polyovular follicles. For the total number of 780 eggs there were 739 corpora lutea and 34 ripe follicles, so that there were 7 more eggs than follicles, either unruptured or luteinized. In other words 0·95 per cent. of the ova seem to have been derived from polyovular follicles.

The planning of the experiment was based on the assumption that ovulation occurs in the Syrian hamster at approximately 1 a.m. (Ward, 1946, 1948*b*). In my observations, however, at 3 a.m. 34 ripe eggs out of 49 mature ova (Tables 1 and 2) had not yet left their follicles. A few ripe follicles may persist, or degenerate through luteinization or atresia; their number is small compared to the number of expelled eggs. We also know that in an animal such as *Mesocricetus* where a great number of eggs are expelled we should not expect all ripe follicles to rupture, as a small percentage may become atretic very late in development, just before rupture.

Ovulation takes place at a somewhat later time than that given by Ward. Her statement is based on 8 ovaries of 8 animals killed between midnight and 1.15 a.m. Only one of 13 ripe follicles had ruptured at midnight. In the short time-interval from 12.30 to 12.45 a.m. Ward (1946) found three ruptured follicles, one

TABLE 3

Age and distribution of eggs in the various segments of the oviduct in the mated animals. The numbers in parentheses are the numbers of fertilized eggs. In column one, m *and* e *mean morning and evening mating respectively*

Estimated ovulation age	Total no. of ova	Mature ova within the ovary	No. of tubal ova	No. of ova in the various segments of the oviduct: Periovarial sac	Infundibulum	Ampulla: Upper	Ampulla: Middle	Ampulla: Lower	Transitional section	Isthmus
0 e	27	20	7	3	—	4	—	—	—	—
6 m	17	—	17	—	—	—	5	12	—	—
1 e	11	—	11	—	—	—	11	—	—	—
2 e	35	—	35	—	3 (1)	26 (1)	6 (3)	—	—	—
8 m	31	—	31	—	—	—	4 (1)	27 (6)	—	—
3 e	10	—	10	—	—	—	10 (6)	—	—	—
4 e	44	—	44	—	—	—	40 (36)	4 (3)	—	—
10 m	23	—	23	—	—	—	—	16 (14)	7 (5)	—
12 m	12	—	12	—	—	—	10 (10)	2 (2)	—	—
10 e	40	—	40	—	—	—	—	38 (38)	1 (1)	1 (1)
16 m	35	—	35	—	—	—	—	10 (10)	9 (9)	16 (15)
16 e	23	—	23	—	—	—	—	—	14 (14)	9 (9)
22 m	23	—	23	—	—	—	—	—	1 (1)	22 (18)
22 e	26	—	26	—	—	—	—	—	5 (4)	21 (17)
28 m	30	—	30	—	—	—	—	—	1 (1)	29 (24)
30 m	14	—	14	—	—	—	—	—	6 (6)	8 (8)
28 e	23	—	23	—	—	—	—	—	—	23 (19)
34 m	24	—	24	—	—	—	—	—	—	24 (23)
36 m	12	—	12	—	—	—	—	1 (1)	4 (4)	7 (5)

TABLE 4

Age and distribution of eggs in the various segments of the oviduct in the unmated controls, grouped according to ovulation age to correspond with the experimental (mated) animals

Estimated ovulation age	Total no. of ova	Mature ova within the ovary	No. of tubal ova	No. of ova in the various segments of the oviduct:						
				Periovarial sac	Infundi-bulum	Ampulla			Transitional section	Isthmus
						Upper	Middle	Lower		
0	21	14	7	—	—	5	2	—	—	—
6	19	—	19	—	—	—	19	—	—	—
2	23	—	23	—	—	23	—	—	—	—
8	17	—	17	—	—	—	17	—	—	—
4	23	—	23	—	—	—	23	—	—	—
10	23	—	23	—	—	—	23	—	—	—
10	24	—	24	—	—	—	3	7	13	1
16	20	—	20	—	—	—	5	5	—	10
16	27	—	27	—	—	—	—	14	4	9
22	22	—	22	1	—	—	—	—	—	21
22	18	—	18	—	—	—	—	—	—	18
28	25	—	25	—	—	—	—	—	—	25
28	19	—	19	—	—	—	—	—	—	19
34	22	—	22	—	—	—	—	—	—	22

in the process of rupturing, and 9 unruptured follicles. Half an hour later (1.00 and 1.15 a.m.) 7 follicles had ruptured, 6 follicles were just rupturing, and 9 were unruptured. In my females killed at 3 a.m. only 15 out of 49 ripe follicles had ruptured. According to Ward's findings (48 follicles) and my data (49 follicles) only 33 out of 97 mature follicles had ruptured between midnight and 3 a.m. At 4 and 5 a.m. there were no more follicles ready for rupturing. All the Graafian follicles at that time showed early signs of atresia. So it seems doubtful whether the high point of ovulation actually lies at 1 a.m.: it seems rather to be an hour or two later. It appears on a basis of 109 ripe or freshly ruptured follicles that the peak of follicular rupture occurs between 1 and 4 a.m., i.e. on the average at 2–3 a.m. This corresponds to the ovulation time of the mouse (Snell, 1941). All the following time data are based on an assumed ovulation time of 3 a.m.

The main data of the results are given in Tables 1–6. A few minor points, however, which cannot be obtained from the tables, are dealt with in the text.

At 3 a.m. 11 of 14 ovulated eggs (combining the mated and unmated groups) were found in the beginning part of the ampulla (Tables 3 and 4), and only three lay in the periovarial space.

Two hours after the assumed time of ovulation, 55 of 58 eggs were found in the ampulla (Tables 3 and 4), while 3 eggs still remained in the infundibulum. One of these ova in which the head of a sperm was visible was in the metaphase of the second maturation division. Since eggs were not found in the infundibulum at a later time, it is concluded that the ova quickly pass the periovarial space and the infundibulum. Approximately 3 hours after ovulation all eggs should have reached the ampulla through which they slowly pass during the next 10–12 hours. The schematic diagram (Text-fig. 1) shows, along with the tortuous nature of the tube, the eggs on their journey through the oviduct, where in the case of a mating they are fertilized.

Table 3 shows that the first fertilized eggs are found 2 hours after the supposed 3 a.m. ovulation; sperm had then penetrated into 5 of 35 ovulated ova. Three of these 5 zygotes lay in the middle part of the ampullar region, while one egg was already fertilized in the cranial portion and another in the infundibulum.

One, 2, and 3 hours after estimated ovulation time 63 unfertilized and 17 fertilized eggs were in the ampulla compared to 3 ova only in the infundibulum. By 4 hours ovulation age the number of unfertilized ova diminishes rapidly. Therefore we can assume that fertilization in the infundibulum is the exception rather than the rule. Eight hours after estimated ovulation time 7 of 31 eggs were fertilized, or just in the process of fertilization; the spermatozoa had partially pierced the membrana pellucida in some, while in others they were in the act of penetrating into the gamete, or had just penetrated. The 7 zygotes lay in the slightly dilated mid- and caudal region of the ampulla (Tables 5 and 6).

Some of the eggs with an estimated ovulation age of 3 hours (Tables 3 and 5) were in the process of fertilization. The head of the sperm was partly transformed into the male pronucleus. The haploid egg nuclei were in the anaphase

TABLE 5

Distribution of the early developmental stages of the golden hamster according to ovulation age. In column one, m *and* e *mean morning and evening mating respectively*

Estimated ovulation age	No. of tubal ova	Unfertilized ova	Fertilized ova	Fertilization: Sperm present	Second meiosis	Pronuclei	1st cleavage	2-cell stage	4-cell stage
0 e	7	7	—	—	—	—	—	—	—
6 m	17	17	—	—	—	—	—	—	—
1 e	11	11	—	—	—	—	—	—	—
2 e	35	30	5	—	5	—	—	—	—
8 m	31	24	7	4	3	—	—	—	—
3 e	10	4	6	—	6	—	—	—	—
4 e	44	5	39	—	10	29	—	—	—
10 m	23	4	19	1	—	18	—	—	—
12 m	12	—	12	3	3	6	—	—	—
10 e	40	—	40	—	1	39	—	—	—
16 m	35	1	34	—	—	34	—	—	—
16 e	23	—	23	—	—	15	6	2	—
22 m	23	4	19	—	—	—	8	9	2
22 e	26	5	21	—	—	9	—	12	—
28 m	30	5	25	—	—	8	5	12	—
30 m	14	—	14	—	2	1	10	1	—
28 e	23	4	19	—	—	7	—	12	—
34 m	24	1	23	—	—	—	—	23	—
36 m	12	2	10	—	1	2	5	2	—

TABLE 6

Distribution of zygotes in relation to the different parts of the oviduct

No. of developmental stages in the various segments of the oviduct	*No. of tubal ova*	*Unfertilized ova*	*Fertilized ova*	*Fertilization:*		*Pronuclei*	*1st cleavage*	*2-cell stage*	*4-cell stage*
				Sperm present	*Second meiosis*				
Periovarial sac	3	3	—	—	—	—	—	—	—
Infundibulum	3	2	1	—	1	—	—	—	—
Ampulla:									
Upper	30	29	1	—	1	—	—	—	—
Middle	86	30	56	3	10	43	—	—	—
Lower	110	36	74	4	14	50	6	—	—
Transitional section	48	3	45	1	3	28	9	4	—
Isthmus	160	21	139	—	2	47	19	69	2
	440	124	316	8	31	168	34	73	2

or telophase of the second meiosis. If the mating occurred 4 or even 6 hours after the presumed ovulation time then 24 out of 31 fertilized eggs with an ovulation age of 10–12 hours had reached the pronucleus stage; in only one egg had the sperm penetrated shortly before death (Table 5). This egg, moreover, was the only one of this extensive material that was still in the beginning phase of fertilization 10 hours after the estimated time of ovulation, and 6 hours after copulation.

With an estimated ovulation age of about 16 hours, copulation having preceded ovulation by about 8 hours, 6 of 14 zygotes in the transitional region still possessed both pronuclei, in 6 others the first cleavage had occurred, and 2 were in the two-cell stage. The 9 eggs lying in the isthmus were in the pronucleus stage (Tables 3 and 5).

Only one embryo with an estimated ovulation age of 22 hours, copulation having occurred about 4 hours after ovulation as a result of a morning mating, lay in the transitional region in the two-cell stage; the remaining eggs had reached the isthmus.

Twenty-two hours following the estimated time of ovulation, in animals mated the evening before, 12 of 21 embryos in the isthmus had already developed to the two-cell stage, while 4 in the transitional region and 4 in the isthmus showed pronuclei; in one the nuclei were in synapsis.

With an ovulation age of 28–30 hours, after a morning copulation, in 6 of the zygotes in the transitional area the first cleavage was occurring while one embryo was at the two-cell stage. In the isthmus 9 eggs were in the pronucleus stage, 9 in the first cleavage stage, and 12 two-cell stages, while 2 eggs were still in the second maturation division (Table 5).

With an assumed ovulation age of 34–36 hours, copulation having followed about 4 hours after ovulation (Table 3), 4 zygotes in the transitional region were at the first cleavage stage. Of the 28 fertilized eggs lying in the isthmus only 2 zygotes were in the pronucleus stage. In one egg there was first cleavage, and 25 embryos were in the two-cell stage; one straggler still in the second meiosis lay in the lower ampulla (Table 5).

DISCUSSION

It is hardly probable that in every case all eggs ovulated (12 per animal) will be fertilized. According to the data it appears that in at most 4 of 38 animals all ovulated eggs were fertilized (Table 1). Twelve or more hours following ovulation 91 per cent. of the eggs ovulated were fertilized, and this may be expected to approximate the maximum for the golden hamster, about 9 per cent. of the eggs shed remaining unfertilized. Among 440 ova, 10 hours following the estimated time of ovulation and 6 hours after copulation, the beginning of fertilization was observed in only one case. From this I conclude that at an increased ovulation age no further fertilization occurs, despite the presence of sperm.

The earliest fertilization of the hamster egg *in vivo* therefore occurs at an ovulation age of 2 hours, while the ability to be fertilized decreases rapidly at about 10 hours after follicular rupture. The optimum time for fertilization for the hamster egg is therefore limited within the range from 2 to 10 hours after ovulation. During this interval the egg stays in the ampulla. It appears that no other place than the ampulla can reasonably be considered as a fertilization site; recently fertilized eggs in variable numbers were found in the middle as well as in the lower region of the ampulla.

The eggs obviously passed through the cranial portion of the ampulla in a short time. Thus at 3 a.m. 9 eggs lay in the beginning portion of the ampulla, while 2 had already appeared in the mid-ampullar region. Two hours later 49 eggs—among them one fertilized—were found in the cranial third of the ampulla, and 6 in the middle third. Two hours later still, that is at an estimated ovulation age of 4 hours, no eggs were found in the cranial ampullar segment. Almost all of the eggs lay in the slightly dilated middle third, while a few had reached the caudal section. From this it is clear that until the peak of fertilization time is reached, between 4 and 12 hours after ovulation, the egg will be found in the mid- and caudal third of the ampulla. There they seem to be in the most favourable position for fertilization, for only one early fertilization stage lies in the cranial third of the ampulla (Table 6). It may be concluded therefore that the union of the egg and sperm in the golden hamster takes place as a rule in the middle and lower third of the ampulla. Nevertheless, fertilization can occasionally occur in the cranial end of the ampulla as well as in the infundibulum.

According to my observations that fertilization of the golden hamster egg occurs chiefly in the middle and caudal third of the ampulla, the problem remains as to how far the morning mating (4 hours after ovulation) possibly influences the position of the fertilization site. At the time of the morning copulation (7 a.m.) practically all eggs expelled 4 hours before had reached the middle and caudal portion of the ampulla. They stayed there until they reached an average ovulation age of 12 hours. Since the spermatozoa need a minimum time of 4 hours to travel up to the ampulla, they would meet ova ovulated about 8 hours previously. The travelling time of the sperms to the region of fertilization follows from the fact that the first fertilized eggs after a morning mating were found in the ampulla at the earliest 4 hours post coitum. The ampullar eggs of females killed before this time are still unfertilized (Tables 3 and 5). If mating took place in the evening before follicular rupture, the first spermatozoa are already in the ampulla at the time of ovulation. Therefore it is surprising not to find all the ova fertilized in the ampulla 3 hours after ovulation. At this time and in spite of the presence of sperms only 11 out of 63 eggs were fertilized (Table 5).

A short time later the proportion of zygotes increases rapidly. This finding supports the idea that the freshly ovulated hamster egg is incapable of being fertilized. The ovum needs to age in order to attain the capacity for fertilization. Table 3 indicates clearly that 4 hours after mating and at an ovulation age of 8

hours, one of 4 eggs lying in the mid-ampullar region was fertilized, and 6 of 27 ova of the caudal third. In the course of the two following hours (6 after copulation, 10 after estimated time of ovulation) even more gametes will be fertilized, whereas the eggs that arrived earlier wander further. Thus I believe that the morning mating does not cause a shift of the fertilization site. On the other hand, eggs of an ovulation age of 12 or more hours meeting the sperm when the ova are already leaving the fertilization site, show a rapidly decreasing fertilization capacity. It becomes zero when the mating occurs 8 or more hours after ovulation (Ward, 1946).

In the pronucleus stage the hamster zygote leaves the fertilization site to appear in the transitional section. We find the first eggs there 10 hours after the hypothetical time of follicular rupture. The last of the ova reach that part of the oviduct at an ovulation age of 16 hours. In the meantime, however, the first 16-hour zygotes have already entered the isthmus. Thus, practically all the eggs have left the ampulla by 16–18 hours after ovulation and are found either in the transitional section, or have already entered the isthmus. Therefore it may be concluded that 20 hours after follicular rupture the greater portion of the female gametes and zygotes had left the transitional section. The presence of spermatozoa in the ampulla and fertilization do not influence the migration tempo in the ampulla, for in the control animals the arrival of the ova within the transitional section occurs between 10 and 16 hours after ovulation. Therefore, it is justifiable to assume that the eggs move relatively rapidly, within a maximum of 6 hours, through the transitional section.

From comparison of Tables 3 and 4 one receives the impression that the eggs may remain in the transitional section longer in the mated animals than in the controls. In the mated animals 10·9 per cent. of the eggs were found in the transitional section against 5·9 per cent. in the control animals. The difference between these values lies possibly outside the margin of error. Furthermore, one must not overlook the fact that 22 to 36 hours after follicular rupture 17 (26·2 per cent.) of 65 eggs were detained in the transitional section; of these 16 were fertilized. In this same time interval in the mated animals, 134 eggs (among them 114 zygotes) had reached the isthmus. In the control series, however, there were no stragglers; all ova had reached the isthmus by 22 hours. This apparent contradiction is supported by the fact that a smaller proportion of the eggs (11·3 per cent.) travel rather more slowly through the transitional section than do the principal mass and the unfertilized eggs. There are no data to explain this. It is conceivable that these retarded eggs experience a variation similar to the retarded fertilized rat and guinea-pig egg as described by Blandau & Jordan (1941) and Blandau & Young (1939).

Since very few exact investigations exist on the fertilization site of the mammalian egg, it is difficult to compare these results with others and to classify them critically. The findings of Sobotta, however, verified by Kremer (1924), were of great value in this work. The section of the tube known as the fertilization site of

Sobotta connects with the ampulla and shows many ampullar characters. Consequently one is justified in stating that fertilization in the hamster and the white mouse takes place in essentially identical sections of the ampulla.

In describing fertilization of the rat egg Tafani (1889), Sobotta & Burckhard (1910), and Huber (1915) adhere closely to the presentation given by Sobotta for the mouse. They observed the fertilization site in the rat as a widened vesicular section of the ampulla which compares to the middle third of the ampulla in the golden hamster.

According to Rein (1883), Rubaschkin (1905), and Lams (1913) a similar relationship seems to exist in the guinea-pig. Even though their data are not as exact as would be desirable for comparison, it seems that the union of the two gametes in *Cavia* likewise occurs in the ampulla. When Hammond (1934) spoke of the proximal end of the oviduct as the fertilization site for the rabbit egg the ampulla was meant. From the data given by R. van der Stricht (1911), and by Hill & Tribe (1924), the fertilization in the cat occurs in the proximal third of the tube and the ampulla should likewise be understood.

The temptation is great to take the results based on the investigation of three representatives of the family Muridae, and to assume when the fertilization site is said to be in the ampulla that it is always the same section of the ampulla. One should be cautious about such generalizations until new investigations have brought to light further details of this question. On the basis of present findings it cannot be said that all mammalian eggs are fertilized in the same area (Strauss, 1938, 1954). In the same way neither a family-specific nor even an order-specific fertilization place can be established at the present time.

SUMMARY

1. The middle and caudal third of the ampulla tubae are the regions where most of the eggs of *Mesocricetus auratus* Waterhouse are fertilized.

2. The earliest fertilization resulting from evening matings prior to ovulation (assuming the ovulation peak to be at 3 a.m.) were obtained at 2 hours after ovulation. When copulation occurred the morning after ovulation, fertilized ova were not obtained at 2 hours after mating, but they were at 4 hours.

3. The time of fertilization is most frequently between the 2nd and 10th hour after ovulation. About 91 per cent. of the eggs are fertilized during this period.

4. On the basis of existing data it is not possible to speak of a family-specific or even an order-specific fertilization site for the mammalian egg.

ACKNOWLEDGEMENTS

This investigation was aided by the Wisconsin Alumni Research Foundation and by grants from the American-Swiss Foundation for Scientific Exchange, Inc., and the Swiss Academy of Medical Sciences.

The author wishes to express his profound gratitude to Dr. H. W. Mossman

for his invitation and aid in this study, which was mostly carried out in his laboratory. Furthermore, the author is indebted to Dr. Margaret W. Orsini for some advice, help in the experiments, and for use of certain data.

REFERENCES

Blandau, R. J., & Jordan, E. S. (1941). The effect of delayed fertilization on the development of the rat ovum. *Amer. J. Anat.* **68,** 275–91.

—— & Young, W. C. (1939). The effects of delayed fertilization on the development of the guinea-pig ovum. *Amer. J. Anat.* **64,** 303–29.

Boyer, Ch. C. (1953). Chronology of development for the golden hamster. *J. Morph.* **92,** 1–38.

Graves, A. P. (1945). Development of the golden hamster, *Cricetus auratus* Waterhouse, during the first nine days. *Amer. J. Anat.* **77,** 219–51.

Hammond, J. (1934). The fertilisation of rabbit ova in relation to time. *J. exp. Biol.* **11,** 140–61.

Hill, J. P., & Tribe, M. (1924). The early development of the cat (Felis domestica). *Quart. J. micr. Sci.* **68,** 513–602.

Huber, G. C. (1915). The development of the albino rat, *Mus norvegicus albinus. J. Morph.* **26,** 247–358.

Kremer, J. (1924). Das Verhalten der Vorkerne im befruchteten Ei der Ratte und der Maus mit besonderer Berücksichtigung ihrer Nucleolen. *Z. mikr.-anat. Forsch.* **1,** 353–90.

Lams, H. (1913). Étude de l'œuf de cobaye aux premiers stades de l'embryogenèse. *Archives de Biologie, Liège & Paris,* **28,** 229–323.

Rein, G. (1883). Beiträge zur Kenntnis der Reifungserscheinungen und Befruchtungsvorgänge am Säugetierei. *Arch. mikr. Anat.* **22,** 233–70.

Rubaschkin, W. (1905). Über die Reifungs- und Befruchtungsprozesse des Meerschweincheneies. *Anat. Hefte, Arb. anat. Inst., Wiesbaden,* **29,** 509–53.

Snell, G. D. (1941). *Biology of the Laboratory Mouse.* Philadelphia: The Blakiston Company.

Sobotta, J. (1895). Die Befruchtung und Furchung des Eies der Maus. *Arch. mikr. Anat.* **45,** 15–93.

—— & Burckhard, G. (1910). Reifung und Befruchtung des Eies der weißen Ratte. *Anat. Hefte, Arb. anat. Inst., Wiesbaden,* **42,** 435–97.

Strauss, F. (1938). Die Befruchtung und der Vorgang der Ovulation bei Ericulus aus der Familie der Centetiden. *Biomorphosis,* **1,** 281–312.

—— (1954). Das Problem des Befruchtungsortes des Säugetiereies. *Bull. schweiz. Akad. med. Wiss.* **10,** 239–48.

van der Stricht, R. (1911). Vitellogenèse dans l'ovule de Chatte. *Archives de Biologie, Liège & Paris,* **26,** 365–481.

Tafani, A. (1889). La fécondation et la segmentation étudiées dans les œufs des rats. *Arch. ital. Biol.,* **11,** 112–17.

Venable, J. H. (1946). Pre-implantation stages in the golden hamster (*Cricetus auratus*). *Anat. Rec.* **94,** 105–19.

Ward, M. C. (1946). A study of the estrous cycle and the breeding of the golden hamster, *Cricetus auratus. Anat. Rec.* **94,** 139–62.

—— (1948*a*). The maturation divisions of the ova of the golden hamster. *Anat. Rec.* **101,** 663.

—— (1948*b*). The early development and implantation of the golden hamster, *Cricetus auratus*, and the associated endometrial changes. *Amer. J. Anat.* **82,** 231–76.

(Manuscript received 19:iv:55)

The Initial Formation and Subsequent Development of the Double Visual Cells in Amphibia

by LAURI SAXÉN[1]

From the Zoological Laboratory of the University of Helsinki

WITH ONE PLATE

INTRODUCTION

In 1867 Schultze described the main types of visual cell in the vertebrates, the rod and the cone, and one year later a third type, the double cone. The two first-mentioned types have subsequently been the objects of a large number of investigations; hence their structure, development, and function are largely understood. On the other hand, the double cells have attracted surprisingly little interest. Many writers apparently do not know of their existence, whilst others mention them only as a rarity, not deserving of particular study. Physiologists, too, speak only of rods and cones, without crediting the double cells with any specific function. The very fact, however, that double cells are extremely common in the vertebrates and compose, as a rule, a considerable proportion of the retinal receptors, should be sufficient evidence that they are not an insignificant rarity, and still less artefacts, as has sometimes been alleged.

The present investigation of the formation and development of the double visual cells in amphibians was undertaken in order to elucidate their origin and significance. A previous study (Saxén, 1954) dealt in detail with the development of the visual cells in one of the species here concerned (*Rana temporaria*), and that paper also included a review of the previous literature on the double cells.

MATERIAL AND METHODS

The investigations were carried out on two species of Amphibia, *Rana temporaria* and *Triturus vulgaris*. In addition to adult animals twelve larval stages from hatching to the end of metamorphosis were studied. In what follows the larval stage is given as the age after hatching. A total of 600 animals were investigated.

Various fixing and staining methods were tried. The best results were

[1] *Author's address*: Zoological Laboratory, University of Helsinki, Helsinki, Finland.

[J. Embryol. exp. Morph. Vol. 4, Part 1, pp. 57–65, March 1956.]

obtained with formol-alcohol or Kolmer's 'cold-blooded' fixative and subsequent staining with Heidenhain's azan or Held's molybdic-acid haematoxylin. Sections were made in the frontal plane, the thickness varying between 5 and 10 μ.

In the tests for glycogen either Carnoy's fluid or freeze-drying fixation was used, and either Bauer's polysaccharide reaction or the PAS staining. The results were checked with the salivary test.

In the colchicine experiments the larvae were kept for two days in colchicine diluted to 1:2000, after which they were fixed in Bouin's solution and stained with sulphurous acid fuchsin (Feulgen).

RESULTS

1. *The double visual cells of adult animals*

The double visual cells of both the species under investigation belong to the classical 'double cone' type. They are composed of a principal component, resembling the single cone, and an accessory component fused with the myoid of the latter (Text-fig. 1D). The accessory component consists of a fairly thin outer segment, a cone-shaped ellipsoid, and a paraboloid, which does not stain by the usual histological methods. The oil droplet peculiar to the *Ranidae* was in *Rana* invariably found in the ellipsoid of the principal component of the double cells, never in the corresponding part of the accessory component. In *Triturus* no oil droplet was found in the single cones, nor in either component of the double cells. The latter always contained two nuclei attached to each other. In both species all double cells were identical; transitional forms were never encountered. The separate accessory cones described by earlier investigators (van Genderen-Stort, 1887; Rozemeyer & Stolte, 1931) were not observed, except in cases where the plane of section separated the two components.

In this connexion it should be recalled that the principal and accessory components stain differently (Saxén, 1953). With Heidenhain's azan the outer segments of the former stain red, like those of the single cone, whilst the outer segments of the latter stain blue, like those of the rod.

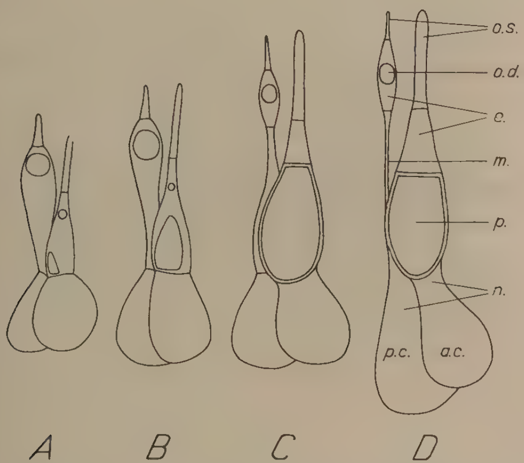
In *Rana* the double cells constituted 10 per cent. of all visual cells, whilst the corresponding figure for *Triturus* was 20.

2. *The initial formation of the double visual cells*

In *Rana* the earliest double cells were observed 5 days after hatching. In the central part of the retina a few double cell progenitors were then seen, consisting of the components shown in Text-fig. 1A. The principal component was identical with the single cone at the same stage of development, but the accessory component was entirely different, most closely resembling a young rod with its thick myoid and long outer segment. The youngest forms displayed only two adjacent cells, the inner segments of which were attached to each other, but in some older cells the accessory component contained a bright basal area, the anlage of the

paraboloid. In both components, just as in the single cell progenitors, an oil droplet was invariably present in the ellipsoid.

In *Triturus* double cells were first encountered at the same stage and site as in *Rana*. As appears in Text-fig. 2A, the two components differed considerably from each other, the most striking difference being the situation of the oil droplet with regard to the ellipsoid.



TEXT-FIG. 1. Development of the double visual cell of *Rana temporaria*. The cell at 5, 7, and 28 days after hatching (A, B, C) and the double cell of an adult animal (D). *a.c.* accessory cone; *e.* ellipsoid; *m.* myoid; *n.* nucleus; *o.d.* oil droplet; *o.s.* outer segment; *p.* paraboloid; *p.c.* principal cone. ×1300.

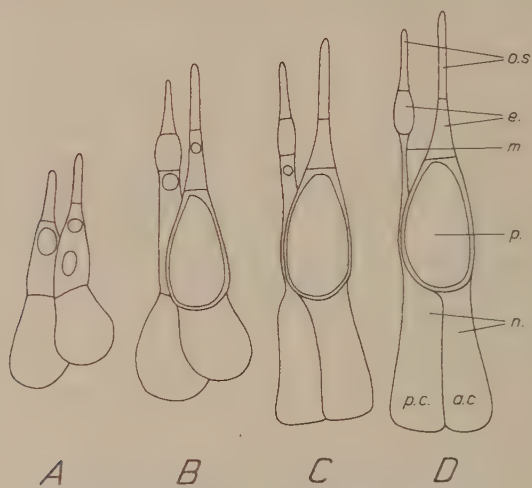
In both species the cells in question were relatively few in number at this stage of their formation, and always situated in the central area of the retina. At the periphery, where the visual cells were younger, neither double cells nor any cell forms that could be regarded as their precursors were found.

It was found in colchicine experiments that during ontogenesis the periphery of the retina, i.e. its undifferentiated part, is the site of division of the visual cells. At the developmental stage described above (4 to 6 days after hatching), mitoses occur only in this area, whilst the differentiated cells of the central part of the retina are no longer capable of division.

It should be emphasized that at the stage when double cells are first observed in the retina, the single visual cells of both the species here concerned are already differentiated, rods and cones being clearly distinguishable. The single rods and cones thus develop earlier than the double visual cells, and progenitors of the accessory components cannot be found before the double cells are formed.

3. *The development of the double visual cells*

Soon after their formation the double cells undergo certain changes which may provide a key to their nature. In both the species studied the accessory component contained for a short time both a distal oil drop and the anlage of a basal paraboloid. After a few days the oil droplet was found to shrink, however, and disappear. This always occurred simultaneously with the disappear-



TEXT-FIG. 2. Development of the double visual cell of *Triturus vulgaris*. The cell at 5, 7, and 28 days after hatching (A, B, C) and the double cell of an adult animal (D). *a.c.* accessory cone; *e.* ellipsoid; *m.* myoid; *n.* nucleus; *o.s.* outer segments; *p.* paraboloid; *p.c.* principal cone. $\times 1300$.

ance of this droplet from the single rods. In *Rana* the oil droplet is maintained in the principal component of the double cone, but in *Triturus*, the single cone of which contains no droplet in adult animals, the droplet disappears from the principal component; this occurs, however, later than in the accessory component and simultaneously with the disappearance of the oil droplet from the single cone. The development of the principal component was found to be like that of the single cone, there being no appreciable difference either morphologically or with regard to staining properties. The development of the accessory component, on the other hand, was different in many respects, and essentially the same in the two species. The next section deals in greater detail with the development of the paraboloid, but certain features characteristic of the development of the accessory component should be discussed in this connexion. Simultaneously with the formation of a clearly defined, dark-staining ellipsoid in the single visual cells, the corresponding segment of the accessory component

developed into an indistinctly defined, faintly staining body. Furthermore, in *Triturus* the droplet due to disappear was located in the ellipsoid of the accessory component, but proximally to this in the principal component. The outer segment of the accessory component, which also differed from the light-sensitive part of the principal component with regard to staining properties, was throughout ontogenesis longer and thinner than the latter.

In both the species investigated a significant decrease in the number of double cells occurred. Immediately after their formation about one-fifth of the visual cells of *Rana* were double cells, whereas in adult animals only one-tenth were of this type. The corresponding figures for *Triturus* were one-third and one-fifth.

4. *The paraboloid*

As mentioned above, the anlage of a basal paraboloid was discernible in the accessory component soon after the formation of the double cells; later this developed into a large flask-shaped body in the inner segment. It did not stain by any of the ordinary methods, but when various glycogen and polysaccharide reactions were tried after specific fixation, the paraboloid always stained intensely. Salivary tests revealed that a substance reacting like glycogen was involved. A glycogen reaction was observable in the paraboloid of the accessory component immediately after its formation. The oil droplet of the visual cells never gave any glycogen reaction.

The paraboloid, which was formed at the end of the first week after hatching, was found gradually to grow into an extensive body filled with a homogeneous substance reacting like glycogen; in both the species here concerned its final shape was constant. In *Rana* the shape of the paraboloid corresponds to its name, the vitreous end being convex and the choroidal end being plane. In *Triturus*, on the other hand, it most nearly resembles an ellipsoid in shape, both ends being convex. It was found that neither illumination nor nutritional conditions influence the shape of the paraboloid or its glycogen content (Saxén, 1955).

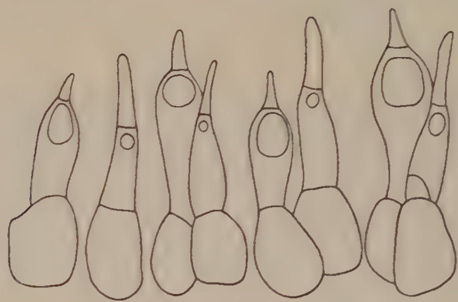
In the single visual cells of these species no paraboloid was found.

DISCUSSION

On the basis of the present results an attempt will be made to form an opinion on the origin of the double visual cells. With regard to this point two conflicting theories have previously been advanced: Levi (1901) assumed that these cells are the result of an incomplete cell division, whereas Detwiler & Laurens (1921) referred them to a fusion of two visual cell progenitors. The present results undoubtedly constitute evidence in favour of the latter of these views. No double-cell progenitors were observed prior to the differentiation of rods and cones, and when the first double cells occur in the central part of the retina, cell division is no longer taking place in this site. In this connexion mention may also be made of the theory according to which the double cells are artefacts.

The latest adherents of this hypothesis are Rozemeyer & Stolte (1931), who stated that these cells are optical illusions referable to the partial coincidence of two adjacent cells. Apart from much other evidence, the fact that the accessory component is never found alone in the retina should be sufficient proof that this theory is erroneous.

All previous investigators have regarded both components of the cell type here concerned as photopic receptors. This is clearly indicated by the name double cone. In view of the present results it cannot be taken for granted, how-



TEXT-FIG. 3. Visual cells of a frog tadpole 5 days after hatching. The long outer segment, the small oil droplet, and the thick base of the myoid are typical of the rods and the accessory components of the double visual cells. The principal components are identical with the single cones. *c.* cone; *d.v.c.* double visual cell; *r.* rod. (Saxén, 1954.) ×1500.

ever, that the cells called double cones are composed of two cone components. In no previous investigation has the present writer found anything to prove this view, which hitherto has not been doubted, whereas the results presented above seem to constitute evidence to the contrary. Firstly, it should be observed that the 'accessory cone' does not occur alone in the retina and that there is no close resemblance between this and either of the main visual cell types of the species here concerned. The shape of the outer segment, the vaguely defined ellipsoid, and the extensive paraboloid are characteristic only of the accessory component; hence there are no grounds for assuming any intimate relationship between this and the single rod or cone. On the other hand, certain features of the accessory component and its development seem to indicate that it is more like a rod than a cone. At the stage of its formation the structure of the accessory component greatly resembles that of a rod at the same stage of development (Text-fig. 3). In both species, moreover, the oil droplet disappears simultaneously from the accessory component and from the single rod. It should be emphasized that the simultaneous occurrence of the oil droplet and the paraboloid, and the results

of glycogen tests, show that the paraboloid does not develop from the oil droplet as has been assumed (Howard, 1908; Moroff, 1922). The difference in the staining properties of the two components also argues in favour of the view that the accessory component is a kind of rod rather than cone. Furthermore, some of Sverdlick's (1954) observations seem to indicate that the cells in question cannot be exclusively photopic. He investigated the number of visual cells in teleosts living at different depths and found that the retinae of surface fishes contained mostly single cones; at greater depths these were to an increasing extent replaced by rods, but also by double visual cells. The fact that the double cells increase in number in dimly illuminated surroundings seems to suggest that the double 'cones' cannot be purely photopic cells, but are rather combined photoreceptors with a wide spectrum and capable of adaptation to highly varying illumination. The present writer believes, therefore, that the name 'double cone' used by previous investigators and in textbooks for the cell type here concerned is ill-founded. On the other hand, the name perhaps explains why this cell type has attracted so little attention amongst physiologists. It conveys the impression that a pure photopic receptor is concerned which, in the light of the duplicity theory, would be of minor interest.

Although the function of an organ or organism is seldom explainable by morphological investigations alone, a brief survey should be given of the evidence on which the present-day understanding of the function of the double visual cells ('double cones') must be based.

1. The structure of the principal component of the double visual cell is always identical with the single cone of the same species and reacts, at least photomechanically, in the same way (van Genderen-Stort, 1887).

2. The structure of the accessory component shows no definite correspondence with either main visual cell type and does not occur alone in the retina. Certain features of its structure and development are strongly indicative of its being a kind of rod.

3. From each double cell two dendrites lead to the inner layers of the retina. According to Cajal (1893) they end at different depths in the outer plexiform layer. There are some indications that they connect with different bipolars (Walls, 1942).

4. In the accessory component of the double visual cell there is an extensive, homogeneous body filled with glycogen, the shape and glycogen content of which are constant (Müller, 1926; Saxén, 1955).

5. Double cells are found in almost all the vertebrates, the higher mammals perhaps excepted. Their relative number is often high, even as high as that of the single cones (Walls, 1942).

From these facts certain conclusions may be drawn. Point 5 clearly shows that the cell type in question must have a specific activity of its own and that it cannot be regarded as an accidental phenomenon or an anomaly any more than as an artefact. The observations summarized as point 4 regarding the constant shape

and size of the paraboloid argue against the view that it is a nutritional organ and that the accessory component has only metabolic significance (Howard, 1908; Franz, 1913). The present writer is an adherent of Müller's (1926) theory that the paraboloid is an optical, lens-like organ. The accessory component must, then, be regarded as a perceptive element and not as a metabolic component or supporting cell.

The present physiological conclusions are based only on embryological and morphological observations and cannot therefore be accepted without reserve. Furthermore, they afford no explanation of the co-ordination and interrelationship between the two components of the double cell. The obviously significant role of this cell type in retinal function should, however, be emphasized, and it is to be hoped that physiologists will direct greater attention than previously to this cell, which apparently constitutes a separate type amongst the photoreceptors of the vertebrates.

SUMMARY

The investigation deals with the development and structure of the double visual cells of two amphibians, *Rana temporaria* and *Triturus vulgaris*. The double cells of these species, which belong to the 'double cone' type described by previous investigators, are encountered later in development than the single visual cells. They are formed through the fusion of two visual cell progenitors. The results support the theory previously advanced by the present author that the accessory component is a rod-like receptor. Thus the name 'double cone' gives an erroneous impression both with regard to the origin of this cell, and probably also with regard to its function. The glycogen droplet in the accessory component, the paraboloid, is apparently a lens-like organ. The function of the double cell is still, however, open to speculation.

ACKNOWLEDGEMENTS

This investigation has been aided by a state grant and by a grant from the Finnish Medical Society 'Duodecim'.

REFERENCES

CAJAL, S. RAMÓN Y (1893). La rétine des vertébrés. *Cellule*, **9**, 119–257.

DETWILER, S. R. (1943). *Vertebrate photoreceptors*. New York: The Macmillan Company.

—— & LAURENS, H. (1921). Studies on the retina. Histogenesis of the visual cells in *Amblystoma*. *J. comp. Neurol.* **33**, 493–508.

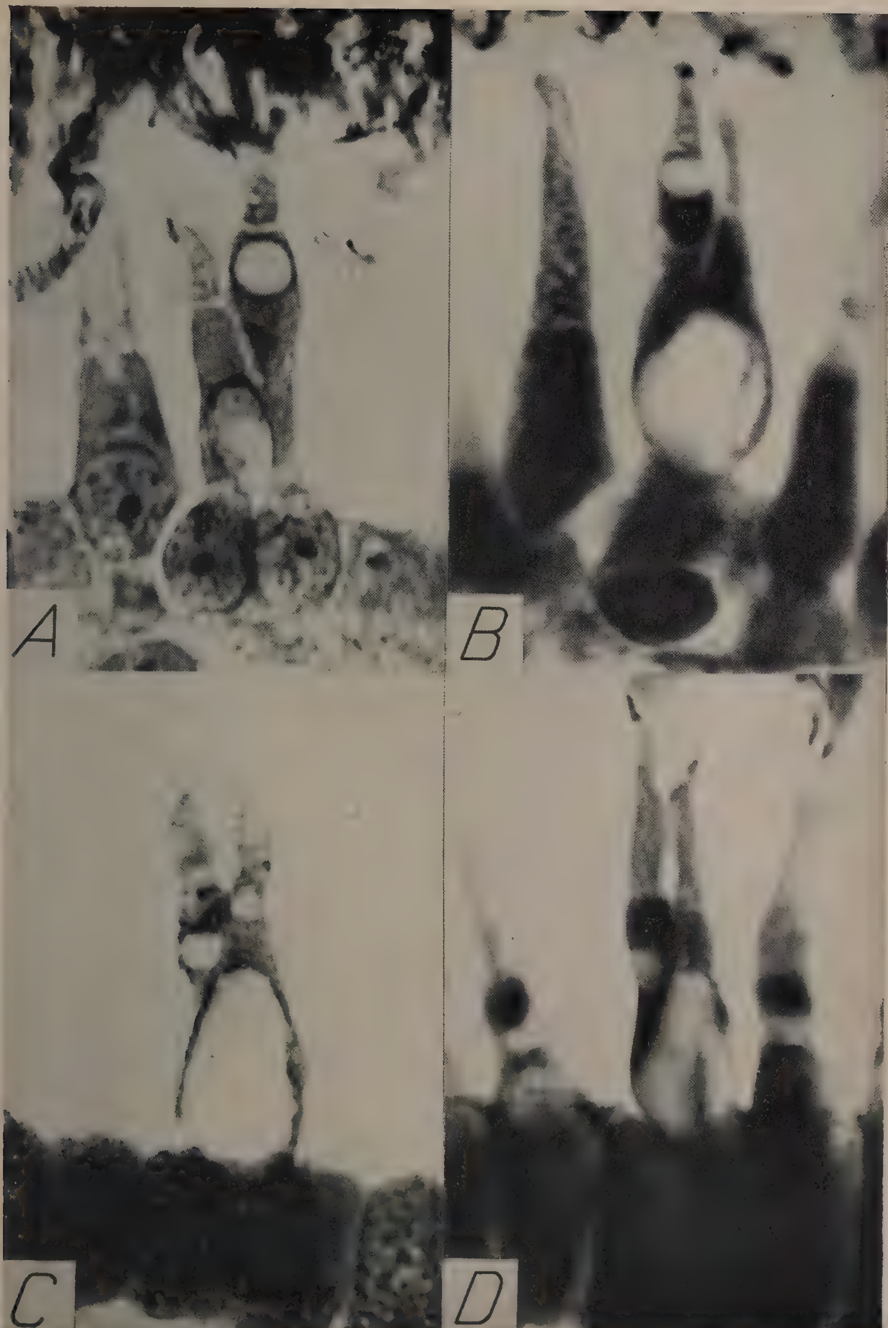
FRANZ, V. (1913). Sehorgan. *Oppels Lehrb. vergl. mikr. Anat. Wirbelt.* **7**, 1–417.

VAN GENDEREN-STORT, A. G. H. (1887). Über Form und Ortsveränderungen der Netzhautelemente unter Einfluß von Licht und Dunkel. *v. Graefes Arch. Ophthal.* **33**, 229–92.

HOWARD, A. D. (1908). The visual cells in vertebrates, chiefly in *Necturus maculosus*. *J. Morph.* **19**, 561–631.

LEVI, G. (1901). Osservazioni sullo sviluppo dei coni e bastonici della retina degli Urodeli. *Sperimentale*, Anno **54**, 6, 521. (Cited from Detwiler, 1943.)

MOROFF, T. (1922). Cyto-histogenese und Bau der Stäbchen und Zapfen der Retina bei Anuren. *Anat. Anz.* **55**, 316–22.



L. SAXÉN

Experiments on Neuromery in *Ambystoma punctatum* Embryos

by BENGT KÄLLÉN[1]

From the Tornblad Institute for Comparative Embryology, Lund, and the Department of Zoology, Washington University, St. Louis, Mo.

WITH TWO PLATES

INTRODUCTION

In a series of papers the present author has discussed the nature and significance of the transverse bulges of the central nervous system of vertebrates, long since known as neuromeres. Their formation and further development have been described and it has been shown that they are signs of a proliferation pattern, made up of transverse bands of high mitotic activity alternating with bands of low activity. It has also been demonstrated that the neuromeric pattern is preceded by another pattern of a similar nature, mirrored in the formation of the so-called proneuromeres, and is followed by a third pattern, giving rise to the postneuromeres or transverse bands of migration areas. For further details of these investigations, the reader is referred to Bergquist & Källén (1954).

An unsolved question is the nature of the factors that cause the patterning of the mitoses and therefore also the bulging of the brain wall to form neuromeres. As far as the present author knows, no investigations on this problem have been published.

The main points to discuss in the present paper are the possible influence from the substratum and the possibility of the existence of influences between different parts of the central nervous system. From the papers of others (Adelmann, 1930, 1934, 1937; Lehmann, 1926; and von Aufsess, 1941), we know that the substratum plays an important role in the modelling of the neural tube, especially of its bilaterality. No observations on the state of segmentation of the brain were made by these authors, however.

MATERIAL AND METHODS

Eggs of *Ambystoma punctatum*, collected in the vicinity of St. Louis, Mo., and in Tennessee, were used. The eggs were washed in 2 per cent. KOH, rinsed

[1] *Author's address*: Tornblad Institute for Comparative Embryology, Biskopsgatan 7, Lund, Sweden.

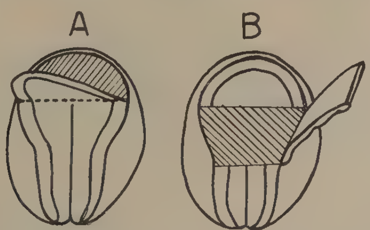
[J. Embryol. exp. Morph. Vol. 4, Part 1, pp. 66–72, March 1956]

in sterile water, and placed in a 0·5 per cent. solution of sodium sulfadiazine and 0·02 per cent. streptomycin in distilled water. The egg membranes were removed and the eggs were transferred to a 10 per cent. dilution of the salt solution described by Flickinger (1949). The operations were then made in a concentrated solution of the same fluid, and the embryos were left in it until wound healing occurred. They were then transferred to the 10 per cent. solution again. All operations were made under aseptic conditions with the help of glass needles and hair loops. The details of the operations will be described later. When the embryos had reached a stage where neuromery was distinct in control embryos, they were fixed in Bouin's fluid, embedded in paraffin, cut in various directions into 10 μ sections, and stained with Delafield's hematoxylin, usually followed by a faint eosin staining. Wax-plate reconstructions were built of some of the series.

RESULTS

1. *Extirpation of the substratum under the brain anlage*

These operations were made at Harrison's stage 13 or 14. The extent of the operations is shown in Text-fig. 1 A, B. A flap was cut out in the neural plate and



Text-fig. 1. Schematic drawings showing the extent of the extirpation experiments. A flap was cut out in the neural plate, either in the archencephalic anlage (fig. A) or in the rhombencephalic anlage (fig. B). The substratum under the flap was cut away and the flap was replaced again.

lifted up, and the substratum was removed under it. The flap was then replaced, and it usually healed in very well. The substratum was extirpated either under the future archencephalon (Text-fig. 1A) or the future rhombencephalon (Text-fig. 1B).

Twenty-two embryos were fixed after extirpations of the substratum under the archencephalon anlage. Of these, 20 were histologically good and 14 were reconstructed. In all cases archencephalic neuromeres were distinct, though a certain variation in number occurred depending on whether or not the optic evaginations had developed. This latter point has been discussed in detail by Bergquist & Källén (1955). Plate 1, fig. A, is a section through one specimen in which the archencephalic neuromeres are distinctly seen.

Eighteen embryos were fixed after excision of the substratum under the

rhombencephalon. Thirteen of these were histologically good, and 5 of the transversely cut series were reconstructed. As is apparent from Plate 2, fig. G, rhombomeres were distinctly developed.

The operation wound in the archenteric roof was filled by mesoderm cells, but the archenteric roof and the notochord were absent in the gap at the time of fixation. No regeneration of these structures had thus occurred.

2. *Transplantation of a piece of the neural plate with or without substratum to the ventral side of an older host*

For these operations the neural plate in stages 13–15 from the rostral end of the neural groove and some distance caudalwards, approximately representing the anlage of the rhombencephalon, was transplanted to the ventral side of a host of stage 18–19 (Text-fig. 2). Either the substratum was included in the

TEXT-FIG. 2. Schematic drawing showing the extent of the transplantation experiments. A piece of the neural plate, approximately corresponding to the rhombencephalic anlage, was cut out in a 13–15 stage embryo. The piece was transplanted with or without corresponding substratum to the ventral ectoderm of a host of stage 18–19.

transplant or it was removed. In some cases the transplanted piece degenerated or did not form a neural tube, but in many cases a neural tube was formed and a fairly normal organization occurred. Twenty-one transplantations with substratum were fixed, and 17 of these were histologically good. Eight were reconstructed. Twenty transplantations without substratum were fixed, 15 were good, and 6 were reconstructed.

Of the 8 reconstructed cases of transplantations with substratum, 5 showed a very clear neuromery, even though the general morphology of the tube was abnormal. In Plate 2, fig. H, such a case is shown. The other three cases had a faint but regular and clear neuromery. In some cases that were not reconstructed neuromeres could be seen directly in the sections (Plate 1, fig. B).

In none of the six cases of transplantations without the substratum that were reconstructed could any traces of neuromery be seen. This was also the case in the remainder of the series that were studied in sections. In Plate 2, fig. I, a reconstruction of such a specimen is shown. It should be emphasized that the neural tubes formed after transplantation without substratum were less well differentiated than those formed after transplantation with the substratum, but in some cases a fairly normal tube was obtained (Plate 1, fig. C).

3. *Explantation of the neural plate with or without substratum*

These operations were made at stages 14–15 or 18–19. The anlage of the brain and the spinal cord as far caudally as possible was cut out from the surrounding ectoderm. Either the substratum was removed as completely as possible, or it was left intact. The ectoderm healed over the explant very rapidly, and it developed fairly normally. A neural tube usually developed and the anlage stretched reasonably well.

When the explantation was made with the substratum, the further development of the explant was very good. Of the 9 reconstructed specimens, 5 showed a very clear and distinct neuromery, 3 a fainter but very regular neuromery, and only 1 specimen showed a somewhat irregular and not very clear neuromery. In Plate 2, fig. J, a reconstruction of an explant belonging to this group is shown.

When the substratum was removed before explantation, somewhat varying results were obtained in different series. It was difficult to remove all mesenchyme along the neural plate, and a varying degree of mesodermal organization was obtained. The neural tube usually developed quite nicely (Plate 1, fig. D). In Table 1 a summary of this group of explants is given. Nineteen explants were made and 9 were reconstructed.

TABLE 1

Embryo number	*Mesoderm organization*	*State of neuromery*
A 81	Much mesenchyme, somites developed	Distinct neuromeres
A 71	Much mesenchyme, somites indicated	Distinct neuromeres
A 66	Rostrally much mesenchyme, somites indicated	A few neuromeres
A 73	Much mesenchyme, no somites	Neuromeres visible but caudally irregular
A 78	Rostrally much mesenchyme, caudally very little	Rostrally distinct neuromeres, caudally none distinct
A 68	As A 78	Rostrally neuromeres, caudally none
A 70	As A 78	Rostrally some irregular neuromeres, caudally none
A 83	Little mesenchyme	Hardly any neuromeres
A 69	Little mesenchyme	No neuromeres

Distinct neuromeres were thus present only in cases where much mesoderm had been left at the operation, and a tendency to somite formation was apparent. In three cases much mesoderm had been left rostrally, but very little caudally, and the neuromeres present were developed in the rostral region. In two cases only very few mesoderm cells could be seen, and the neuromery was practically absent. In Plate 2, figs. K and L, reconstructions illustrating this point are shown.

In order to show the different degree of neuromeric formation in the explants

where the substratum was and was not included, two microphotographs are shown in Plate 1, figs. E and F. The regular neuromeres of the explant with substratum (fig. E) contrast with the irregular and faint bulges of the explant without substratum (fig. F).

DISCUSSION

It was observed that the neuromeres develop much more distinctly in the explants and transplants in which the substratum was included than in those in which the substratum was removed, though the organization of the neural tube was sometimes very good after the latter operations. The preparations with substratum may have shown a greater tendency to stretch than those without substratum, but the difference was not very conspicuous, as is apparent from the illustrations. A greater stretching of the tube would, if anything, lessen the distinctness of the neuromeres; hence this factor cannot play any role in the outcome of the experiments.

The substratum thus seems to play an active part in the formation of the neuromeres. This conclusion, however, seems to be contradictory to the results of the substratum extirpation experiments (section 1 of Results). A way of explaining this apparent contradiction is to suppose that in the latter experiments the influence from the parts of the archenteric roof lying rostral and caudal to the wound affected the neural tube in the defective region. The role of the substratum in the formation of neuromeres would then be of a general nature, and the periodic spatial arrangement of the mitoses in the neural tube would be due to special properties of the central nervous system. The segmentation of the nervous system is thus not secondary to mesoderm segmentation, even if the former is in part caused by a factor emanating from the substratum.

As we know that the basis of neuromery is a segmental pattern of intensified proliferation, it seems reasonable to conclude that the general effect of the substratum which these results suggest may be that of a mitotic stimulation in the neural tube. Previously Wagner (1949) and Overton (1950) have described a mitogenetic effect of one embryonic tissue on another, and it seems quite probable that a similar effect exists between the substratum and the neural tube in the stages in question.

From the transplantation experiments it appears that neuromeres can develop in the rhombencephalon even in the absence of adjacent parts of the neural tube.

SUMMARY

1. When the substratum was removed from under the archencephalic or rhombencephalic anlagen of the neural plate of *Ambystoma punctatum* embryos, the neuromeres in these regions developed normally.

2. When the rhombencephalic part of the neural plate was transplanted to the ventral ectoderm of an older host together with substratum, neuromeres

developed in the transplant. When the transplantation was made without the substratum, no distinct neuromeres appeared.

3. When the neural plate was explanted together with the substratum, neuromeres were formed in the explant. When the explantation was made without the substratum, neuromery was either absent or, when some mesoderm had been left behind, very irregular.

4. It is concluded that the substratum plays an important role in the formation of the neuromeres. The latter are not secondary to a mesodermal segmentation. The influence from the substratum is probably a general mitotic stimulation.

ACKNOWLEDGEMENTS

The experiments were performed in the Department of Zoology, Washington University, St. Louis, Mo., during the author's stay there as Rockefeller Research Fellow. The author is indebted to Dr. Hamburger for help with the operation technique. The reconstructions were made at the Tornblad Institute, and were financed by the Swedish Medical Research Council.

REFERENCES

Adelmann, H. B. (1930). Experimental studies on the development of the eye. III. The effect of the substrate ('Unterlagerung') on the heterotopic development of median and lateral strips of the anterior end of the neural plate of Amblystoma. *J. exp. Zool.* **57**, 223–81.

——(1934). A study of cyclopia in *Amblystoma punctatum* with special reference to the mesoderm. *J. exp. Zool.* **67**, 217–81.

—— (1937). Experimental studies on the development of the eye. IV. The effects of the partial and complete excision of the prechordal substrate on the development of eyes of *Amblystoma punctatum*. *J. exp. Zool.* **75**, 119–227.

Aufseß, A. von (1941). Defekt- und Isolationsversuche an der Medullarplatte und ihrer Unterlagerung an Triton alpestris und Amblystoma-Keimen, mit besonderer Berücksichtigung der Rumpf- und Schwanzregion. *Roux Arch. EntwMech. Org.* **141**, 248–339.

Bergquist, H., & Källén, B. (1954). Notes on the early histogenesis and morphogenesis of the central nervous system in vertebrates. *J. comp. Neurol.* **100**, 627–60.

—— —— (1955). The archencephalic neuromery in Ambystoma punctatum. An experimental study. *Acta anat.* **24**, 208–14.

Flickinger, R. A., jr. (1949). A study of the metabolism of amphibian neural crest cells during their migration and pigmentation in vitro. *J. exp. Zool.* **112**, 465–84.

Lehmann, F. E. (1926). Entwicklungsstörungen in der Medullaranlage von Triton, erzeugt durch Unterlagerungsdefekte. *Roux Arch. EntwMech. Org.* **108**, 243–82.

Overton, J. (1950). Mitotic stimulation of amphibian epidermis by underlying grafts of central nervous tissue. *J. exp. Zool.* **115**, 521–60.

Wagner, G. (1949). Die Bedeutung der Neuralleiste für die Kopfgestaltung der Amphibienlarven. Untersuchungen an Chimaeren von Triton und Bombinator. *Rev. suisse Zool.* **56**, 519–620.

EXPLANATION OF PLATES

Plate 1

Fig. A. Transverse section through an embryo (A 109) operated according to Text-fig. 1A. The archencephalic neuromeres appear clearly. Magnification ×65.

Fig. B. Sagittal section through a transplant (A 61) from an operation according to Text-fig. 2, in which the substratum was included. The neuromeres are distinct. Magnification ×60.

FIG. C. Transverse section through a transplant (A 33) from an operation according to Text-fig. 2, in which the substratum was removed at the operation. The neural tube is well differentiated. Magnification ×80.

FIG. D. Transverse section through an explant (A 89) without the substratum, showing the well-differentiated neural tube. Magnification ×80.

FIG. E. Horizontal section through an explant (A 39), showing distinct neuromeres. The substratum was included in the explantation. Magnification ×60.

FIG. F. Horizontal section through an explant (A 70) without substratum. No distinct neuromeres can be seen. Magnification ×65.

PLATE 2

FIG. G. Wax-plate reconstruction of the brain of an embryo (A 12) operated according to Text-fig. 1B; lateral view. The rhombomeres are distinct. Magnification ×30.

FIG. H. Wax-plate reconstruction of a transplant in an embryo (A 55) operated according to Text-fig. 2 with the substratum included. The neuromeres are distinct. Magnification ×30.

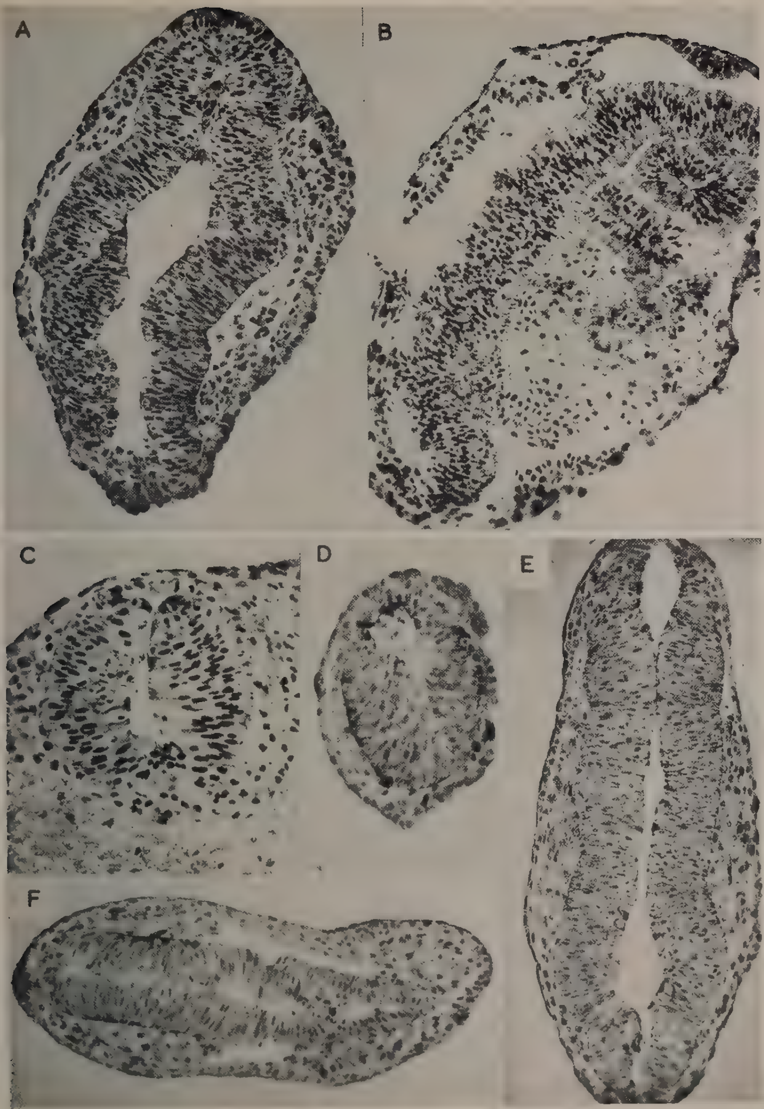
FIG. I. Wax-plate reconstruction of a transplant in an embryo (A 88) operated according to Text-fig. 2 without the substratum. No neuromeres can be seen. Magnification ×30.

FIG. J. Wax-plate reconstruction of an explant (A 50) with the substratum. The neuromeres are regular and distinct. Magnification ×30.

FIG. K. Wax-plate reconstruction of an explant (A 66) after removal of substratum. In this case relatively much mesoderm was formed, and faint neuromeres developed. Magnification ×30.

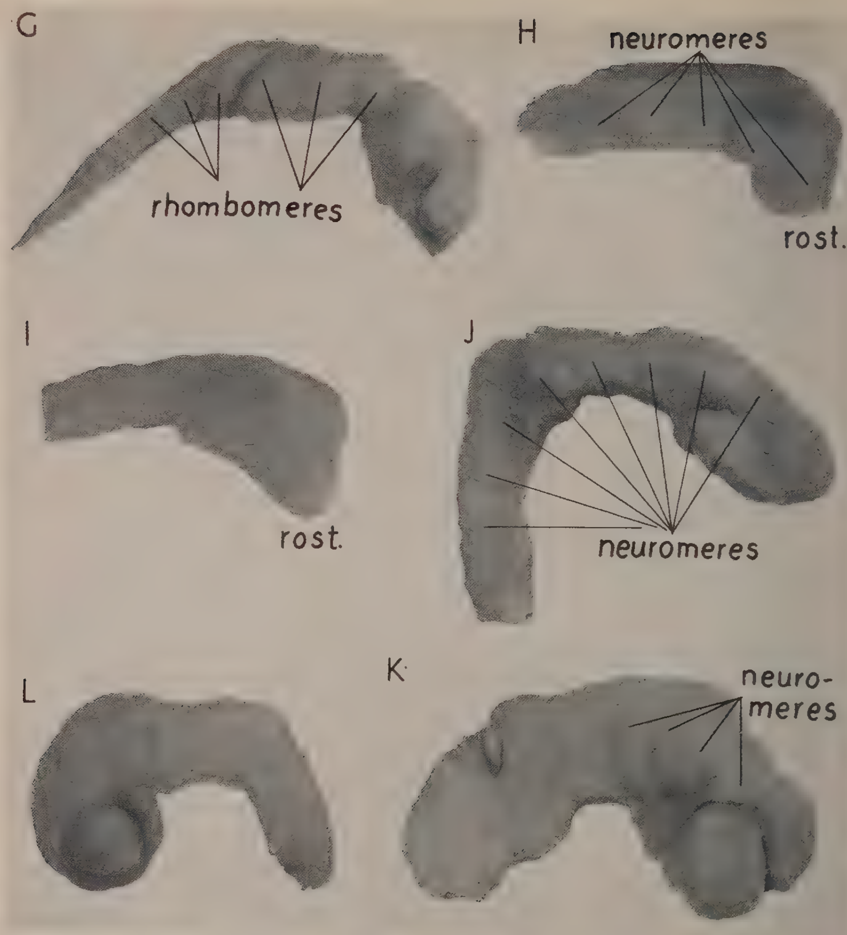
FIG. L. Wax-plate reconstruction of an explant (A 83) without substratum. Only very little mesoderm was formed, and no distinct neuromeres can be seen. Magnification ×30.

(Manuscript received 23:vi:55)



B. KÄLLÉN

Plate 1



B. KÄLLÉN

Plate 2

Selective Inhibition of Cleavage in Different Regions of the Frog Egg by Sulphydryl Inhibitors

by LUCENA J. BARTH[1]

Department of Zoology, Columbia University, New York City

INTRODUCTION

THAT the materials for protein synthesis in the frog egg must come from yolk is indicated by the constancy of total nitrogen during development (Gregg & Ballentine, 1946) and the fact that the egg can develop with no outside source of organic or inorganic materials. When and where in the developing egg new proteins arise, and what are the mechanisms which control the rate and direct the specificity of such syntheses, are problems which are beginning to occupy increasing numbers of investigators using several methods of attack—immunological, enzymological, electrophoretic, and incorporation of labelled amino acids, for example.

Brachet (1940), using histochemical methods, described a change in the distribution of sulphydryl proteins coincident with grey crescent formation. In the newly-laid egg of *Triton* or *Pleurodeles* Brachet found the sulphydryl proteins to be restricted to a small spot centred about the maturation figure near the animal pole. This picture changed during the first few hours after fertilization. Although Brachet noted a fair amount of individual variation, in all cases part of the sulphydryl proteins were displaced toward the equator. At the 2-cell stage the most typical situation was that part of the SH substances remained confined to the animal pole while the rest formed a crescent or a ring enlarged on one side. At the 4- and 8-cell stages Brachet was able to determine in the species he used that the favoured side of the egg with regard to sulphydryl protein content was the one where the blastomeres were smaller, i.e. probably the presumptive dorsal lip side of the equatorial zone.

In the course of experiments in which eggs of *Rana pipiens* at stages from just after fertilization to the 2-cell stage were exposed to various inhibitors, we observed that the compound *p*-chloromercuribenzoic acid (CMB), an effective inhibitor of sulphydryl (SH) groups, reacted with various regions of the egg in a selective way. When CMB-treated eggs reached the gastrula stage it was found that the dorsal lip of the blastopore had formed as usual in a normally cleaved

[1] *Author's address*: Department of Zoology, Columbia University, New York 27, N.Y., U.S.A.

[J. Embryol. exp. Morph. Vol. 4, Part 1, pp. 73–92, March 1956]

zone between pigment and yolk. The pigmented animal pole cells, however, had not cleaved beyond the 32–64-cell stage.

This inhibitor appeared to us to offer a tool for the examination of differences in the SH content or availability of SH groups in different protein fractions of the egg. Our programme became (1) to localize the SH groups at different stages of development, using CMB sensitivity as a marker; (2) to examine the effects of other compounds known to react with SH groups; (3) to examine possible mechanisms by which the SH inhibitors might act to produce differential inhibition of cleavage; and (4) to examine the SH content of various protein fractions whose distribution within the egg is to some extent known. It seemed possible that the regional differences in CMB sensitivity might reflect differences in the distribution or molecular state of different proteins within the living egg.

EXPERIMENTAL

I. *The effect of CMB on early development*

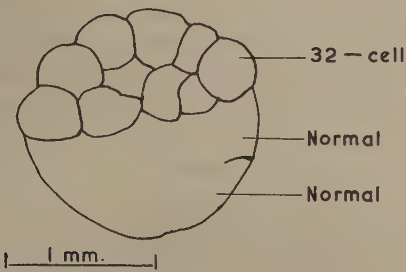
A. *Continuous exposure from the 2-cell stage to gastrulation*

Jelly was removed by means of watchmakers' forceps from eggs of *Rana pipiens* before first cleavage. At the 2-cell stage twenty eggs were pipetted into each of several stender dishes containing 15 ml. of a 1×10^{-5} M solution of CMB in 0·1 Ringer's solution from which phosphate and carbonate had been omitted, with the pH adjusted to 7·2–7·3. The stenders, together with controls in which O·1 Ringer's at the same pH was used instead of the inhibitor, were placed at 20° C. on an inclined rotating table to provide for mixing and uniform exposure to the solutions. When the controls had reached gastrula stages (S 10 or 11 of Shumway, 1940) the appearance of control and treated eggs was observed.

The appearance of the treated eggs is represented diagrammatically in Text-fig. 1, where it can be seen that although the marginal zone cleaved normally and a dorsal lip is forming, the pigmented cells at the animal pole were arrested at the 32–64-cell stage. The yolk cleaved normally below the dorsal lip, but was somewhat inhibited on the presumptive ventral side. In the large animal pole cells the pigment appeared to have withdrawn, giving a lighter grey appearance, but there was no indication of cytolysis. The normal marginal zone region extends higher toward the animal pole on the dorsal lip side than on the presumptive ventral lip side.

A clutch-to-clutch variation in susceptibility of eggs was observed but there was no deviation from the characteristically selective regional effects of the mercury compound. For example, in a clutch showing relatively less inhibition, the animal pole cells might cleave to stage 8 size (mid-cleavage) with marginal zones normal and dorsal lips equivalent to stage 11. Even less inhibited embryos sometimes developed farther and at stage 14 looked normal save for the presence of a group of cytolysing cells at the anterior end of the neural plate. This region

represents the position at neurulation of cells earlier located near the animal pole of the egg.



TEXT-FIG. 1. Diagrammatic representation of the typical appearance of a gastrula resulting from treatment of an egg during early cleavage with 1×10^{-5} M *p*-chloromercuribenzoic acid.

B. *Susceptibility of eggs at different stages of development to CMB*

1. *The development of eggs during continuous exposure to CMB beginning at different stages of development.* Jelly was removed from about 300 eggs, which then were kept at room temperatures of 21–25° C. until they reached the

TABLE 1

Regional differences in susceptibility of eggs during continuous exposure to 1×10^{-5} M p-chloromercuribenzoic acid beginning at different stages of development

Stage at which eggs were immersed in CMB	*Stage of development of regions after 18–24 hours, 20° C.*			
	0·1 Ringer's controls	*CMB-treated*		
		Animal	*Vegetal*	*Marginal zone*
2-cell	S10	32-cell	S10	S10
32–64-cell	S10	32–64-cell	S10	S10
S8, mid-cleavage	S11	S8	S10–11	S10–11
S9, late	S13+	Eggs died at late S9		
S10	S14	Eggs died at S10+ after some exogastrulation		

desired stages of development. Groups of eggs at different stages were transferred into the CMB solution (15–20 eggs per 15 ml. solution). After 18 to 24 hours at 20° C. the eggs were examined. The observations are organized in Table 1. The differential inhibition of animal pole cells appears to be characteristic of eggs exposed during the earlier stages of cleavage, whereas late blastulae and early gastrulae are uniformly and immediately inhibited in all regions and soon die.

This experiment as set up does not permit a decision as to the possibility of

increased permeability with stage of development, nor does it strictly delimit the exposure period. In a separate experiment, it was determined that eggs exposed at first cleavage develop normally if they are removed from the inhibitor, washed and transferred to 0·1 Ringer's at any time up to 6–8 hours' exposure depending on clutch-to-clutch variations in susceptibility. The fact that animal pole cells proceed to cleave from 2 to 32 cells might therefore merely

TABLE 2

Regional differences at successive stages of development in susceptibility of eggs exposed for brief intervals to 1×10^{-4} M p-chloromercuribenzoic acid

Stage of eggs at time of exposure	*Duration exposure in minutes*	*Appearance of regions after development at 20° C. in 0·1 Ringer's after exposure*		
		Animal	*Vegetal*	*Marginal zone*
20 min. after fertilizing	30	32-cell	No cleavage	No cleavage
60 min. after fertilizing	30	32-cell	No cleavage	No cleavage
60 min. after fertilizing	10	16–256-cell	No cleavage	Dorsal side = 16–256-cell
2-cell	30	32-cell	No cleavage	No cleavage
2-cell	10	S8	Cleaved in grey crescent	Dorsal side = S10
2-cell	20	256-cell	Cleaved in grey crescent	Dorsal side = S10
4-cell	20	64–128-cell	No cleavage	No cleavage
32-cell . . .	20	Variable 64–128-cell, S8	Cleaved in grey crescent	Dorsal side = S10
32-cell . . .	20	S8	S8	S8
64-cell . . .	20	256-cell	256-cell	256-cell
64-cell . . .	20	256-cell	Cleaved in grey crescent	Dorsal side = S10
128-cell . . .	20	256-cell	128-cell	256-cell
128-cell . . .	10, 20, 30	256-cell	256-cell	256-cell
256-cell . . .	5, 10, 20, 30	256-cell	256-cell	256-cell
S11+ . . .	20	S11+, surface eroded	S11+	S11+, surface eroded
S12	20	Die at abnormal S14	S14	
S13	10, 20, 30	Die at S15		

reflect a slow rate of penetration of the mercury compound at this early stage. It might also mean that the inhibitor does not reach the deep-lying nuclei or mitotic apparatus of the large cells formed by early cleavage.

2. *The development of eggs after rapid penetration of a high concentration of CMB at different stages of development.* In order to introduce the CMB rapidly at a given and limited stage of development, the concentration of the inhibitor was increased tenfold to 1×10^{-4} M and the duration of exposure reduced to 10, 20, or 30 minutes. It proved impossible to fertilize eggs that had been stripped into the inhibitor and washed several times after penetration of the CMB. The stages exposed therefore ranged from 20 minutes after fertilization to stage 13. The relative inhibition by CMB of various regions of the egg

is shown in Table 2. In this as well as subsequent tables 'number of cells' is to be interpreted as 'estimated number of cells'.

Eggs exposed from the 64-cell stage on are found to cleave normally in all cell layers beneath the surface, indicating that the mercury actually is bound by more superficial cells whereupon their further cleavage is inhibited. While there is too much variation to permit final conclusions (due possibly to uneven penetration during the brief interval of exposure), the trend indicated by Table 2 may be described as follows. Just after fertilization, the cells at the animal pole are

TABLE 3

Regional differences at successive stages of development in susceptibility of eggs chilled to retard development during penetration of 1×10^{-5} *M p-chloromercuribenzoic acid*

Eggs exposed to CMB 1×10^{-5} M, 8° C. during development from	*Appearance after development at 20° C. in 0·1 Ringer's*			
	Control 0·1 Ringer's	*CMB-treated*		
		Animal	*Vegetal*	*Marginal zone*
60 min. after fertilizing to 16-cell	S11+	Cleaved	Uncleaved	Uncleaved
2-cell to 32-cell	S11+	32–64-cell	S11	Dorsal lip = S11
2-cell to 32-cell	S12	S8	Cleaved in grey crescent	S9+
32-cell to 64-cell	S12	32–64-cell	Cleaved in grey crescent	S10–S11
32-cell to 128-cell	S13–	128-cell	Cleaved in grey crescent	S10–S12
64-cell to 256-cell	S12	256-cell	Cleaved in grey crescent	S10–S11
128-cell to S8	S14	128-cell	Normal	S11–S12+

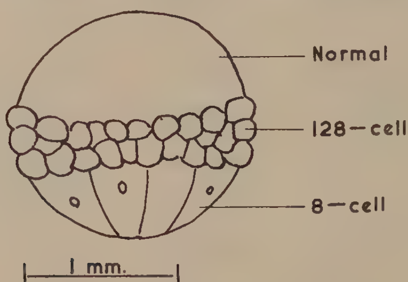
less susceptible to inhibition by CMB, while by 32–64 cells the 'protected' region has shifted to the marginal zone between animal and vegetal hemispheres. At the same time the yolk on the grey crescent side of the egg becomes less susceptible to mercury inhibition. These selective differences in susceptibility to mercury are abolished by the 128–256-cell stage, whereafter the entire egg is inhibited, and at gastrula and neurula stages exposure to CMB results in almost immediate arrest of cleavage in the surface cells of all regions.

3. *Effect of slow penetration at lower concentration of CMB during limited phases of development.* Because the penetration of 1×10^{-4} M CMB into eggs exposed for short intervals appeared to be variable, a better method of introducing the inhibitor into eggs at limited stages of development was devised. Development can be retarded for 18 to 24 hours by keeping eggs at different stages at 8° C., during which time the CMB at 1×10^{-6} M diffuses into the eggs. The eggs then are washed free of unbound inhibitor and transferred to 0·1 Ringer's for development at 20° C. The chilling does not produce developmental abnormalities during the period observed in controls kept in 0·1 Ringer's.

Observations on this type of experiment are recorded in Table 3, from which it may be concluded that for at least 60 minutes after fertilization the animal pole region is less susceptible to CMB than the yolk and marginal zone, which fail to cleave at all. By the 2-cell stage the protected or unsusceptible area is located on the grey crescent side of the egg, both in the yolk and pigmented marginal zone, and it is the cells around the animal pole which are inhibited. Eggs exposed at 32-, 64-, and 128-cell stages develop to gastrular stages with blastoporal lips, but much of the animal hemisphere fails to cleave. The data confirm the results of the preceding section in showing a shift in the area protected from the effects of CMB from the animal pole to the dorsal marginal zone. This shift occurs between 60 minutes after fertilization and the 2-cell stage.

II. *The effect of iodoacetamide on eggs exposed at early cleavage*

The selective inhibition of animal pole cells by CMB might be due to greater permeability of these cells to the inhibitor. Evidence against a regional permeability difference was obtained by using another inhibitor of SH groups, iodo-



TEXT-FIG. 2. Diagrammatic representation of a typical 'gastrula' resulting from treatment of an egg during early cleavage with $2–2{\cdot}5 \times 10^{-4}$ M iodoacetamide.

acetamide, which reacts rapidly though not exclusively with SH groups (Chinard & Hellerman, 1954; Barron & Singer, 1945). Iodoacetic acid, potassium ferricyanide, and potassium permanganate also were tested.

Experiments with iodoacetamide gave the most informative results, in that this SH inhibitor too had selective effects on different regions of the egg, but these were precisely the opposite to the results obtained with CMB. As Text-fig. 2 shows, cleavage of the entire animal cap proceeds normally in iodoacetamide, while yolk cleavage is arrested. The marginal zone cells fail to cleave in iodoacetamide and bulge out as a grey, swollen, but not cytolysing band of cells overhanging the uncleaved yolk and overlapping the animal cells above.

The inhibition of yolk cleavage and of the cells in the marginal zone is a uniformly characteristic result of iodoacetamide treatment (Table 4). In several

experiments a normal blastoporal lip was formed, despite the inhibition of the cells above and below it.

The diametrically opposite effects of CMB and iodoacetamide are not readily explained on the basis of differential permeability of the cells composing animal, vegetal, and marginal zones. More plausible is the tentative hypothesis that the two inhibitors have affinities for different groups located at opposite poles of the

TABLE 4

Regional differences in susceptibility of eggs at early cleavage to iodoacetic acid, iodoacetamide, potassium permanganate, and potassium ferricyanide

Abbreviations are as follows: IAA = iodoacetic acid; IACET iodoacetamide. N = normal cleavage to same stage as 0·1 Ringer's controls; I = inhibition of cleavage; + = present; — = absent.

				Effect on development			
				Treated			
Inhibitor	*pH*	*Molar concentration*	*Control 0·1 Ringer's*	*Animal*	*Vegetal*	*Marginal*	*Dorsal lip*
IAA . .	5·2	$5{\cdot}0\times10^{-3}$	S10	128-cell	N	N	+
IAA . .	5·2	$2{\cdot}5\times10^{-3}$	S11	S8	N	S9	—
IAA . .	5·2	$5{\cdot}0\times10^{-3}$	S12	S8	N	S9	—
IACET . .	6·1	$2{\cdot}5\times10^{-4}$	S12	N	N	N	+
IACET . .	6·1	$2{\cdot}5\times10^{-4}$	S12	N	I	I	+
IACET . .	6·1	$2{\cdot}5\times10^{-4}$	S12	N	I	I	—
IACET . .	5·8	$2{\cdot}5\times10^{-4}$	S10	N	I	I	+
IACET . .	5·8	$2{\cdot}0\times10^{-4}$	S12+	N	I	I	—
IACET . .	5·8	$2{\cdot}5\times10^{-4}$	S11	N	I	I	—
IACET . .	5·7	$2{\cdot}5\times10^{-4}$	S12	N	I	I	+
IACET . .	5·7	$7{\cdot}5\times10^{-5}$	S10+	N	I	I	—
IACET . .	6·0	$7{\cdot}5\times10^{-5}$	S10+	N	I	I	—
$KMnO_4$.	7·1	$5{\cdot}0\times10^{-4}$ $1{\cdot}0\times10^{-4}$	S10+	Partial exogastrulation; no regional differences in cleavage			
$KMnO_4$.	7·1	$5{\cdot}0\times10^{-5}$ $2{\cdot}5\times10^{-5}$	S10	Cytolysed at S9 to S10			
$K_3Fe(CN)_6$.	7·1	$1{\cdot}0\times10^{-3}$ $1{\cdot}0\times10^{-4}$	S11	General inhibition at S9			
$K_3Fe(CN)_6$.	7·1	$5{\cdot}0\times10^{-4}$	S12	Abnormal blastopore lips; partial exogastrulation			

egg. The mechanism of inhibition of SH groups by iodoacetamide and CMB differ (Barron & Singer, 1945) in that the former compound is an alkylating agent, substituting the H of the SH group by an alkyl group, while the mercury compound forms a mercaptide, in which the H of the SH group is substituted by a metal. The two inhibitors differ also in their affinities for SH groups, the CMB attacking groups not touched by the other inhibitor. The selective affinity of the two inhibitors for different regions of the egg is presumptive evidence for the location of different kinds of SH compounds in different regions of the egg.

Potassium ferricyanide and potassium permanganate, although capable of oxidizing SH groups, are not specific for these groups, hence the general, non-selective inhibition that occurred with these compounds is neither surprising

nor informative. Less severe inhibition in some experiments with potassium permanganate resulted in partial exogastrulation, as Bellamy (1919) also reported.

A difference between the effects of iodoacetic acid and of iodoacetamide was detected (Table 4). The results of iodoacetic acid treatment resemble a mild CMB inhibition such that the marginal zone cleaves normally, while the animal pole cells are arrested at 128-cell to mid-cleavage stages.

III. *Reversal of CMB inhibition by thioglycollate and cysteine*

Since neither iodoacetamide nor CMB may be regarded as entirely specific for SH groups (iodoacetamide reacts also with amino groups, and CMB could conceivably exert its effect through competitive inhibition by the benzoic acid moiety, Chinard & Hellerman, 1954), it is necessary to bring about reversal of

TABLE 5

Reversal of p-chloromercuribenzoic acid inhibition by thioglycollate and cysteine

Hours exposure to CMB	*Controls in 0·1 Ringer's throughout*	*Appearance of treated eggs after subsequent development in*		
		0·1 Ringer's	*Thioglycollate*	*Cysteine*
9·75	S11	64–128-cell	Animal cells = 64–128; dorsal lips = S10–S11	. . .
9	S10	256-cell	Animal cells = S8 and S10 dorsal lips in 50%	. . .
7	S10	Animal cells = S8; dorsal lips = S10	. . . . .	High % of normal S10
		Appearance of eggs after development in		
Hours exposure	*0·1 Ringer's*	*CMB*	*CMB + thioglycollate*	*CMB + cysteine*
22	S11+	Animal cells inhibited; dorsal lips = S10–S11	. . . . .	S11+
21	S11	128-cell	S11	. . .
21	S11	Animal cells inhibited; dorsal lips = S10–S11	S11	. . .

the inhibition by the addition of mercapto compounds before concluding that the effect of the inhibitor is mediated by SH groups.

Eggs at the 2-cell stage were immersed in CMB, 1×10^{-5} M. At times varying from 7 to 10 hours eggs were removed from the inhibitor, washed through several changes of either 0·1 Ringer's or 0·25 per cent. sodium thioglycollate, pH 7, and transferred for further development to either 0·1 Ringer's or thioglycollate. Control eggs were subjected to the same manipulations but remained always in 0·1 Ringer's. When the controls reached stage 10 or stage 11 the treated eggs were examined for comparison of the CMB effect with and without

subsequent substitution of thioglycollate. In another group of experiments the ability of cysteine (1×10^{-2} cysteine-HCl, pH adjusted to 7) to reverse the CMB inhibition was examined. The results shown in Table 5 indicate that both compounds are able to reverse the CMB inhibition. The reversal is not complete and is not sustained, however, because the eggs cytolyse during gastrulation. Only simultaneous exposure to CMB and thioglycollate (0·9 CMB + 0·1 2·5 per cent. thioglycollate to keep concentrations constant) permitted gastrulation to proceed beyond dorsal lip formation. Although this result could be explained by reaction of the two compounds in solution and not within the egg, it proves that the inhibitory effect of CMB is mediated by SH groups. Thioglycollate and cysteine alone permitted entirely normal development at the concentrations used for protection and reversal with CMB.

In experiments similarly designed to test the reversibility by thioglycollate of the iodoacetamide and iodoacetic acid effects, the iodoacetic acid effect was found to be partially reversible, whereas thioglycollate had no effect on iodoacetamide-inhibited eggs. This difference is consistent with the similar morphological effects of iodoacetic acid and CMB (both reversible by thioglycollate), as opposed to the different effect of iodoacetamide on the egg (irreversible by thioglycollate). It is possible therefore that iodoacetamide produces its inhibitory effects on cleavage of yolk cells by combining with groups other than SH, amino groups, for example.

Other experiments designed to attempt protection against CMB through the use of substrates and metabolites whose synthesis or availability might be limited by inhibition of SH-dependent enzymes gave only negative results. (1) With the hypothesis that the marginal zone might be protected from CMB inhibition by its high yolk-protein content, dialysed yolk proteins were provided along with the CMB solution. The animal pole cells still were inhibited. (2) Pretreatment of eggs with sodium succinate, sodium ATP and DPN before exposure to CMB did not alter the inhibitory effect of the mercury compound. (3) A modification (Cohen, 1954) of the Le Page solution for glycolysis, including phosphate buffer, nicotinamide, $MgCl_2$, Na_2ATP, DPN, and cysteine-HCl, proved somewhat toxic and failed either to prevent or reverse the CMB inhibition. Nicotinamide was found to inhibit yolk cleavage.

IV. *Respiration of inhibited eggs*

In considering the mechanisms through which SH inhibitors could affect cleavage at least two possibilities come to mind. The groundwork was laid by Rapkine for the now generally accepted view that the oxidation and reduction of sulphur play an important role in mitosis (Brachet, 1950, chapter 5; Barron & Seki, 1952) and that the mitotic apparatus contains bound sulphur (Mazia & Dan, 1952). It is known furthermore that for many enzymes involved in the oxidation of fats, carbohydrates, and proteins at least a fraction of their SH

groups must be maintained in reduced form for catalytic activity (Chinard & Hellerman, 1954). Although the frog egg can cleave for a time in the absence of oxygen, the lactic acid debt thus accumulated has come through the glycolytic pathway that includes SH-dependent enzymes—triose-*P*-dehydrogenase, for example. If either CMB or iodoacetamide combines with such enzymes a lowered rate of respiration would be expected, followed eventually by arrest of cleavage. Cleavage could also be arrested by combination of the SH reagent used in low concentrations with non-protein SH groups, with an expected increase in respiration (Barron, 1951). Experiments were made therefore to determine whether changes in respiration accompanied the effects produced on the development of eggs in CMB and iodoacetamide.

A series of experiments in which eggs at 32-cell stage were placed in the two inhibitors in Warburg flasks and respiration measured during penetration of the compounds gave data that indicated no significant differences in the oxygen consumption of iodoacetamide-treated eggs and 0·1 Ringer's controls.

The respiration of eggs in 0·1 Ringer's *after* penetration of the two inhibitors varied only slightly from that of 0·1 Ringer's controls, and in directions explainable in terms of the regional differences in susceptibility to the inhibitors. In CMB, which selectively inhibits cleavage of the animal pole cells that have the highest rate of respiration in the egg (Sze, 1953), the oxygen consumption was slightly lower than that of 0·1 Ringer's controls. After iodoacetamide treatment, which inhibits vegetal and marginal zones, respiration was approximately equal to that of 0·1 Ringer's controls. This situation is to be expected on the basis of the small contribution to total oxygen consumption made by the yolk cells normally.

V. *Localization of protein-bound SH groups in different protein fractions of the egg*

A. *Soluble cytoplasmic proteins*

The normal cell division of the marginal zone and the yolk hemisphere that occurs at concentrations of CMB which severely inhibit animal hemisphere cells could result from a lack of SH groups in yolk and marginal regions; from protection by proteins containing SH groups but not involved in cell division; or from an excess of SH groups such that low concentrations of CMB sufficient to combine with all the animal-pole SH groups are not adequate to saturate all the SH groups in the yolky and marginal regions. If the SH groups differ in various regions of the egg such differences might provide clues to the nature of and interactions between various proteins as development proceeds.

A study of the distribution of SH groups in various protein fractions was begun by separating the proteins soluble in weak saline-phosphate buffer from the yolk proteins and applying the nitroprusside test as a qualitative test for the presence of free SH groups. Since large quantities are required to give a detectable reaction with nitroprusside, ovarian eggs and hatched tadpoles were used

for the tests. Non-protein SH and protein-bound SH had to be distinguished in the extracts, and in addition a fractionation of the soluble extract with ammonium sulphate was carried out. All operations were performed at 1–4° C.

1. *Comparison of soluble and yolk fractions.* Ten ml. ovary plus 5 ml. 0·1 Ringer's were homogenized. The brei, pH 6·1, was centrifuged 30 minutes at 18,000*g* and the supernatant fluid containing soluble protein and non-protein components was recentrifuged and saved for test. The residues from the first centrifuging were washed, with homogenizing, and recentrifuged in a total of 25 ml. of 0·1 Ringer's. To the washed residues 10 ml. of 0·1 Ringer's and 90 ml.

TABLE 6

Results of nitroprusside tests for SH groups in various fractions obtained from ovarian eggs and hatched embryos

Expt. no.	*Source of extract*	*Fraction*	*Strength of reaction with nitroprusside*
1	Ovaries	Soluble	++
		Soluble, ppt. with sat. $(NH_4)_2SO_4$	++
		Soluble, supernatant after pptn. with sat. $(NH_4)_2SO_4$	++
		Yolk	—
2	Ovaries	Soluble, whole extract	+
		Ppt. at 24% sat. $(NH_4)_2SO_4$	+
		Ppt. at 56% sat. $(NH_4)_2SO_4$	++
		Ppt. at 100% sat. $(NH_4)_2SO_4$	—
3	Hatched embryos S20	Soluble, whole extract	++
		Ppt. at 24% sat. $(NH_4)_2SO_4$	+
		Ppt. at 56% sat. $(NH_4)_2SO_4$	++
		Ppt. at 100% sat. $(NH_4)_2SO_4$	slight

of 10 per cent. NaCl in M/15 phosphate buffer, pH 7·2, were added. The tarry black mixture was transferred as completely as possible to a ten-Broeck grinder and homogenized thoroughly. The homogenate was centrifuged 25 minutes at 18,000*g* and the extracts were pooled. Aliquots of this extract containing yolk proteins then were precipitated in 20 volumes of distilled water and the large precipitates obtained after 15 minutes' centrifuging at 1,000*g* were dissolved in 0·1 Ringer's saturated with ammonium sulphate. The total volume of precipitate and solution was 22 ml. The soluble extract saved from the first part of the procedure was made to 22 ml., and 17·6 g. ammonium sulphate were added to saturation. Five ml. aliquots from each fraction (soluble and yolk) were removed for the nitroprusside tests. In order to separate protein-bound SH from non-protein SH another 5 ml. aliquot of the soluble fraction was saturated with ammonium sulphate and centrifuged for 15 minutes at 18,000*g* to separate the precipitated proteins from the supernatant non-protein components. The results of the nitroprusside tests appear in Table 6, expt. 1.

2. *Fractionation of the soluble proteins.* Ten ml. of ovaries (Table 6, expt. 2)

or 1,600 stage 20 embryos + 1 ml. of 0·1 Ringer's (Table 6, expt. 3) were homogenized in a ten-Broeck grinder with 10 ml. of 1·3 per cent. NaCl adjusted to pH 7·9. The pH of the resultant brei is 6·9 and the salt concentration 0·65 per cent. Aliquots were centrifuged 30 minutes at 18,000*g*, and the supernatant fluid withdrawn from below the floating fat cap by means of a fine pipette. After re-centrifuging for 20 minutes at 18,000*g*, the supernatant extract was divided as follows: 2·5 ml. were removed for a nitroprusside test. This aliquot contains both non-protein and protein-bound SH. To 10 ml. of the extract dry ammonium sulphate was added to give 24 per cent. saturation. After centrifuging, the 24 per cent. precipitable residues were washed with 24 per cent. saturated ammonium sulphate, while the supernate was brought to 56 per cent. saturation with ammonium sulphate. After centrifuging down and washing the residues precipitable at 56 per cent. saturation, the supernatant solution was saturated with ammonium sulphate and the small precipitate collected. As we had observed earlier (unpublished experiments in which the soluble fraction was precipitated in ammonium sulphate at 8 per cent. intervals from 8 to 96 per cent. saturation) three peaks of precipitation were observed. Most of the soluble proteins of the egg precipitate at 56 per cent. saturation, while a smaller quantity come down at 24 per cent. and still less at 100 per cent. saturation. Each of the three residues obtained at 24 per cent., 56 per cent., and 100 per cent. saturation was dissolved in 5 ml. of 0·65 per cent. NaCl, pH 7·9, and saturated by adding 3·6 g. ammonium sulphate for the nitroprusside test.

The nitroprusside tests were carried out according to the method of Anson (1941) in which 5-ml. test solutions are saturated with ammonium sulphate and 5 drops of concentrated NH_4OH plus 5 drops of fresh, aqueous, 4·5 per cent. sodium nitroprusside solution are added.

The results presented in Table 6 show that all the free, protein-bound SH groups of the egg are located in the proteins soluble in weak saline phosphate buffer, i.e. in the extractable cytoplasmic proteins (Gregg & Ballentine, 1946; Sze, 1953). All three fractions separated by ammonium sulphate precipitation contain SH groups. The soluble fraction also includes non-protein SH groups. The yolk as extracted appears to have no free SH groups that will give a positive nitroprusside test.

B. *Yolk proteins*

1. *Whole yolk.* Because the yolk as extracted contained no free SH groups that would react with sodium nitroprusside, an attempt was made to determine the nature of the sulphur bond in yolk. Yolk proteins were obtained from ovarian eggs in the usual way (p. 83, above) by extracting in 10 per cent. NaCl, pH 7·2, defatting the extract and precipitating its proteins in 20 volumes distilled water. The nitroprusside tests were carried out according to the method of Anson (1941) after treating the protein solutions according to Greenstein & Edsall (1940) to denature and/or to split the disulphide bonds.

The yolk proteins as obtained from the egg in presumably native state again lack free SH groups (Table 7). While treatments that denature (guanidine-HCl and urea) release some SH groups, and a small proportion also are released by

TABLE 7

Results of nitroprusside tests for SH groups in native, denatured, and reduced yolk proteins obtained from ovarian eggs

Material added to 1 ml. protein solution	*Probable effect of treatment*	*Strength of reaction with nitroprusside*
1.	Native	−
2. 1·6 g. guanidine-HCl	Denatured	++
3. 1·0 g. urea	Denatured	+
4. 1·6 g. guanidine-HCl + 2 drops 2N NaCN + 2 drops conc. NH_4OH	Denatured + S-S bonds reduced	+++++
5. 2 drops NaCN + 2 drops conc. NH_4OH .	S-S bonds reduced	+

splitting the disulphide bonds of native yolk proteins, by far the largest source of SH groups in yolk resides in a form that can be made available only by denaturation plus subsequent splitting of the disulphide bonds appearing upon denaturation.

2. *Dephosphorylated yolk proteins.* Harris (1946) has shown that the egg contains an enzyme, phosphoprotein phosphatase, that splits inorganic phos-

TABLE 8

Results of nitroprusside tests for SH groups in dephosphorylated yolk proteins

Material added to 1 ml. protein solution	*Probable effect of treatment*	*Strength of reaction with nitroprusside*
1.	Native	−
2. 1·6 g. guanidine-HCl	Denatured	+
3. 1·0 g. urea	Denatured	+
4. 1·6 g. guanidine-HCl + 2 drops 2N NaCN + 2 drops conc. NH_4OH	Denatured + S-S bonds reduced	++++−
5. 2 drops 2N NaCN + 2 drops conc. NH_4OH .	S-S bonds reduced	−
6. 2 drops 0·1 N NaCN + 1·6 g. guanidine-HCl	Removal of metal impurities in guanidine that interfere with test	++

phate from phosphoprotein and presumably functions in making yolk phosphate available to the morphogenetically active regions of the developing egg. Previous work (Barth & Barth, 1954) has shown that when inorganic *P* is released from the yolk-protein complex (S_1–S_2) extracted from the embryo, both S_1 and S_2 become soluble in acetate buffer, pH 4·7. It seemed worth while to investigate whether when phosphate was split off there was any concomitant change in the molecular state of the yolk proteins that would be reflected in a release of SH groups.

A large incubation mixture of all the proteins (enzymes + yolk) extractable in 10 per cent. NaCl, pH of brei 6·9, and precipitated in 20 volumes of water, was made up by homogenizing the precipitates in acetate buffer. After 60 minutes' incubation at room temperatures of 20–23° C. the mixtures were centrifuged at 18,000*g* for 25 minutes. The pooled supernatants containing the now soluble dephosphorylated phosphoprotein and the other yolk protein (S_1) were used for nitroprusside tests. The results (Table 8) show that even after dephosphorylation the SH groups of the proteins are not freely reactive and become so only after denaturation followed by splitting of disulphide bonds.

TABLE 9

Results of nitroprusside tests for SH groups in two protein fractions obtained from yolk

Material added to 1 ml. protein solution	Probable effect of treatment	Nitroprusside reaction by phosphoprotein S_1	S_2
1.	Native	—	—
2. 1·6 g. guanidine-HCl	Denatured	—	—
3. 1·6 g. guanidine-HCl + 2 drops 2N NaCN + 2 drops conc. NH_4OH	Denatured + S-S bonds reduced	+ + + + +	slight

3. *Localization of SH groups within the protein fractions of yolk.* Since we had worked out (Barth & Barth, 1954) an effective method for separating the phosphoprotein of yolk from the other non-phosphate containing protein with which it appears to be combined in extractable yolk, we prepared samples of the two yolk proteins and tested each for SH groups before and after denaturation and reduction of disulphide groups.

The results in Table 9 prove unequivocally that the protein lacking phosphate (S_1) is the source of SH groups in yolk and that phosphoprotein itself is free of SH groups that can be released by denaturation and reduction of the disulphide linkage. The faint colour obtained with the phosphoprotein was due probably to incomplete removal of S_1.

DISCUSSION

When the results of the present experiments with *Rana pipiens* are compared with Brachet's (1940) histochemical studies on eggs of *Triton* and *Pleurodeles*, it is apparent that both methods indicate a rearrangement of sulphydryl-containing compounds before first cleavage. The present experiments, however, reveal that two regions of the egg both may be nitroprusside-positive but may differ qualitatively in their response to low concentrations of the sulphydryl inhibitor CMB. Brachet's picture of the localization in the newly-laid egg of sulphydryl components at the animal pole must be reconciled with the *resistance* of this

region to cleavage inhibition by CMB. Brachet's description of the 2-cell stage, in which part of the nitroprusside-positive materials now are located about the marginal zone in a crescent or ring enlarged on one side, must be compared with the present demonstration that cleavage in the presence of CMB proceeds normally in this ring-shaped region, while animal-pole cells now are inhibited.

The nitroprusside-positive groups remaining at the animal pole at first cleavage appear to belong to constituents involved in cleavage and sensitive to concentrations of CMB that permit normal cleavage of the marginal zone. It may be this type of sulphydryl compound whose continued synthesis during later cleavage and blastulation results in the nitroprusside-positive reaction throughout the entire animal hemisphere obtained by Brachet and in the non-selective inhibition of cleavage by CMB in the entire upper hemisphere noted at late blastula stages in the present experiments.

Inhibition of cleavage by CMB in a region that is nitroprusside-positive, such as the animal pole at the 2-cell stage, is not difficult of interpretation in terms of the role of sulphydryl groups in mitosis. The anomalous situation at the marginal zone, however, requires further analysis. How can one interpret the fact that the marginal zone, which also is nitroprusside-positive at the 2-cell stage, cleaves normally in concentrations of a powerful sulphydryl inhibitor that block the animal pole cells? What is the nature of the sulphydryl components that move down from the animal pole or are synthesized *de novo* at the marginal zone at the time of grey crescent formation?

Brachet considered that his method detected protein-bound sulphydryl groups since he could repeat or prolong the preliminary trichloracetic acid treatment without diminishing the intensity of the reaction. He pointed out, however, that the method itself may denature the proteins so that sulphydryl groups not present in the living egg may be unmasked. The failure of CMB to inhibit cleavage in the marginal zone could conceivably then mean an absence in this region of the living egg of accessible protein sulphydryl groups. Such an hypothesis appears rather dubious, however, in view of the ability of CMB to react with even deep-lying SH groups (Barron & Singer, 1945).

The inhibitor experiments could, on the other hand, be interpreted to mean that the sulphydryl components appearing at the marginal zone differ qualitatively from those remaining at the animal pole; that the marginal zone components are sulphydryl proteins involved in differentiation but not necessary for cleavage. If these proteins are more readily accessible than the sulphydryl proteins of the mitotic apparatus, their presence would serve to protect the latter from inhibition by mercury.

Another possible explanation of the present results would postulate the existence of an excess of sulphydryl compounds at the marginal zone sufficient to protect this region from concentrations of CMB that inhibit less favoured zones. In view of the lack of direct quantitative determinations of either protein-bound or free sulphydryl groups in the living frog egg, one can only speculate

along these lines. The soluble, non-protein SH compounds are more easily attacked than protein-bound SH groups (Barron & Seki, 1952), and therefore at the low concentrations of CMB employed in the present experiments lack of susceptibility may represent the localization of such soluble SH compounds as reduced glutathione and cysteine. Several functions have been demonstrated for these soluble thiols (Barron, 1951). (1) Glutathione and cysteine are important factors in the regulation of the energetic processes of the cell. When this regulatory system is withdrawn an increase in respiration ensues, accompanied by inhibition of cleavage. (2) The reversibility of the oxidation of the soluble thiols provides them with a protective role toward fixed SH groups.

If we visualize a shift of some of the protein-bound SH from animal to marginal regions before first cleavage (Brachet, 1940), and add to this fact the hypothesis that soluble thiols accompany this rearrangement of egg components, another explanation for some of the results obtained in the present experiments suggests itself.

Normal cleavage depends upon both types of thiols (Barron & Seki, 1952). Soluble thiols protect protein-bound thiols against SH reagents such as CMB. The animal cap may lose these protective soluble thiols at fertilization when the well-known rotation of the egg brings about a redistribution of its plasms. The marginal zone, particularly on the presumptive dorsal lip side, might gain soluble thiols, thereby imparting to the protein-bound SH groups a protection against thiol reagents such as CMB. The hypothesis that the dorsal lip and marginal zone of the gastrula possess a relatively high content of reduced thiols is consistent with the demonstrations by Child and by Piepho (both quoted in Brachet, 1950, p. 363) of a high rate of reduction of vital dyes by these regions.

Bellamy (1919) used another thiol reagent, mercuric chloride, among other inhibitors in a demonstration of differential susceptibility during early development of the frog, and his results indicated a shift in susceptibility from animal pole to dorsal lip that is consistent with Brachet's later histochemical localization of SH proteins. It is possible that mercuric chloride dissociates more completely and provides a higher concentration of mercury ions for union with the protein-bound SH groups than does CMB, so that the soluble thiols are inadequate to protect the protein SH from this powerful inhibitor.

The marginal zone is admirably situated in regard to availability of raw materials for the continued synthesis of both soluble and protein-bound SH, since it is the zone in which enzymes concentrated primarily in the animal hemisphere come into contact with the large stores of sulphur, phosphorus, and protein of yolk (S_1 = sulphur source; S_2 = phosphorus source). Adenosine triphosphate, which is utilized in the enzymatic synthesis of glutathione (Waelsch, 1952 *a* and *b*), may be present in greater quantity in the dorsal lip region (Fujii *et al.*, 1951).

If this interpretation of the present experiments can be substantiated, what significance may be attached to a high glutathione content in the dorsal lip and

marginal zone? Waelsch (1952 *a* and *b*) has proposed a possible mechanism for peptide bond synthesis involving the participation of glutathione, ATP, and the enzyme glutamotransferase, and in recent experiments (Rudnick, Mela, & Waelsch, 1954) glutamotransferase was detected in the area opaca, and a relatively high specific activity in the yolk sac epithelium of the chick embryo was measured. These are regions in which intensive protein synthesis occurs. It may be significant in this connexion that Buño (1951) found that in the chick blastoderm the free yolk is entirely negative to the nitroprusside reaction, while the area opaca, where yolk is in contact with the blastodisc, contains an abundance of free SH. The marginal zone of the frog egg may also be a region where conditions for peptide bond synthesis are favoured by high concentrations of ATP, glutathione, and amino acids from yolk. For the specificity of such syntheses we may look toward a possible correlation of Brachet's extensive studies on ribonucleoproteins in the egg with the scheme of Gale & Folkes (1955) which provides for specificity of amino acid incorporation through the participation of specific di- and trinucleotides.

The diametrically opposite effects obtained here with CMB and iodoacetamide are indeed puzzling, particularly in view of experiments reported by Rapkine & Brachet (1951). Working with eggs of the frog, *Triton*, and *Axolotl* and using concentrations of iodoacetamide in the same range as we used but for shorter periods of time, these investigators obtained embryos almost lacking a nervous system but possessing practically normal chorda and somites. Thus in Rapkine and Brachet's experiments with iodoacetamide the mesoderm resists inhibition, while the presumptive neural plate is susceptible to the inhibitor—a situation we found in using CMB but not with iodoacetamide.

The question of why yolk cleavage is normal in concentrations of CMB that severely inhibit cleavage of cells at the animal pole is not readily answered. The working hypothesis used to explain protection of the marginal zone would imply that yolk also has a high content of soluble reduced thiols, an assumption contradicted by the failure of yolk regions of the living gastrula to reduce vital dyes. Norman (1954) demonstrated clearly that in the grasshopper egg the yolk does not contain soluble free thiols, and suggested that the latter are formed as a result of the activity of enzymes located in the embryo itself. It is true that the protein sulphur of yolk may be bound too deeply within the yolk proteins to be accessible to thiol reagents until after denaturation. This possibility is consistent both with Brachet's (1940) observation that in the several species he studied with the exception of the axolotl egg the yolk is negative to nitroprusside even after denaturation, and with our own negative results with the nitroprusside reaction upon extracted yolk proteins prior to denaturation and reduction of the disulphide bonds. These yolk proteins, however, are derived from the platelets and presumably are not involved in cleavage. The thiol groups of the mitotic apparatus of yolk cells, on the other hand, lie deep within the large, platelet-laden cells of early cleavage (a fact easily observed in stained sections), and a time lag

in diffusion of the inhibitor to these deep-lying groups might explain their unsusceptibility.

The mechanism by which CMB inhibits cleavage of animal hemisphere cells has not been investigated. Gregg & Ornstein (1953) reported that CMB used at a higher concentration than in the present experiments produces disruption of the surface coat in explants without causing cytolysis. In the present experiments it was observed that the large, inhibited cells at the animal pole of CMB-inhibited embryos appear to have suffered a loss or withdrawal of pigment from the surface. It is difficult, however, to explain the selective inhibition of cleavage observed in different regions of the egg in terms of differences in sensitivity of the surface coat and cell membranes. More probably the mercury compound inhibits cleavage by combining with elements of the mitotic apparatus and the changes in pigment distribution are secondary to the inhibition of cleavage caused by CMB.

It is notable that although the presence of ATP in the frog egg and of enzymes able to hydrolyse it have been demonstrated conclusively (Brachet, 1950, p. 463; Barth & Jaeger, 1947), evidence has been lacking that ATP is coupled in a causal manner with any developmental process. Ambellan (1955) recently has found an acceleration in rate of neural tube closure in embryos exposed during the blastula stage to solutions of ATP, or of a mixture of AMP and ADP. The mechanism underlying this specific developmental effect of the nucleotides awaits elucidation. *In vitro* studies already have suggested several functions for ATP in the metabolism of the frog egg. The rate of phosphoprotein breakdown from yolk may be controlled by the concentration of ATP (Barth & Barth, 1954), which in turn donates phosphate for the synthesis of nucleic acids (Kutsky, 1950). The possibility now is suggested that ATP in the marginal zone of the frog egg, together with the sulphur-containing protein of yolk, may participate in the synthesis of glutathione and in turn of peptide bonds.

SUMMARY

1. When frogs are exposed during early cleavage to low concentrations of the sulphydryl inhibitor, *p*-chloromercuribenzoic acid, cleavage is inhibited at the animal pole, while the marginal zone and yolk cleave normally. Eggs exposed before first cleavage cleave only at the animal pole, while blastulae and gastrulae are inhibited uniformly in all regions by the sulphydryl reagent.

2. The selective inhibition of animal-pole cells by *p*-chloromercuribenzoic acid is not readily explained on the basis of regional permeability differences or variations in sensitivity of the surface coat to inhibitors, since exposure of eggs to iodoacetamide results in normal cleavage at the animal pole, together with inhibition of cleavage at the marginal zone and in the yolk.

3. The inhibition produced by *p*-chloromercuribenzoic acid is mediated by sulphydryl groups, since the effects of the inhibitor are reversible by thioglycollate and cysteine.

4. When various protein fractions are extracted from eggs or hatched embryos and tested for sulphydryl groups by means of the nitroprusside reaction, the soluble fractions are found to contain freely reactive sulphydryl groups, while the yolk proteins are entirely lacking in reactive protein-bound sulphydryl groups. When the yolk proteins are fractionated into phosphoprotein (S_2) and non-phosphoprotein (S_1), the latter alone is found to contain masked SH groups that become fully reactive only after denaturation plus reduction of disulphide groups.

5. It is suggested that the protein-bound SH groups at the marginal zone are protected against *p*-chloromercuribenzoic acid inhibition by a high content of soluble thiols such as reduced glutathione, which are synthesized at the expense of sulphur-containing amino acids from the yolk protein S_1, under the control of enzymes and nucleotides such as adenosine triphosphate present in the dorsal lip region and marginal zone.

ACKNOWLEDGEMENTS

The writer is grateful to Professor Lester G. Barth for valued criticism and advice during the experiments reported above and the interpretation thereof.

These investigations were made with the aid of a grant (G-3322, c3) from the division of research grants, Public Health Service, Department of Health, Education and Welfare, National Institutes of Health.

REFERENCES

AMBELLAN, E. (1955). *Proc. nat. Acad. Sci. Wash.* (in press).

ANSON, M. L. (1941). The sulphydryl groups of egg albumin. *Journ. gen. Physiol.* **24**, 399–421.

BARRON, E. S. G. (1951). Thiol groups of biological importance. *Advanc. Enzymol.* **11**, 201–66.

—— & SEKI, S. L. (1952). Studies on the mechanism of action of ionizing radiations. *Journ. gen. Physiol.* **35**, 865–71.

—— & SINGER, T. P. (1945). Studies on biological oxidations. XIX. Sulphydryl enzymes in carbohydrate metabolism. *Jour. biol. Chem.* **157**, 221–40.

BARTH, L. G., & BARTH, L. J. (1954). *The Energetics of Development*. New York: Columbia University Press.

—— & JAEGER, L. (1947). Phosphorylation in the frog's egg. *Physiol. Zoöl.* **20**, 133–46.

BELLAMY, A. W. (1919). Differential susceptibility as a basis for modification and control of early development in the frog. *Biol. Bull. Wood's Hole*, **37**, 312–61.

BRACHET, J. (1940). Étude histochimique des protéines au cours du développement embryonnaire des poissons, des amphibiens et des oiseaux. *Archives de Biologie, Liège & Paris*, **51**, 167–202.

—— (1950). *Chemical Embryology*. New York: Interscience Publishers, Inc.

BUÑO, W. (1951). Localization of sulphydryl groups in the chick embryo. *Anat. Rec.* **111**, 123–8.

CHINARD, F. P., & HELLERMAN, L. (1954). Determination of sulphydryl groups in certain biological substances. In *Methods of Biochemical Analysis*, **1**, 1–26.

COHEN, A. I. (1954). Studies on glycolysis during the early development of the *Rana pipiens* embryo. *Physiol. Zoöl.* **27**, 128–41.

FUJII, R., UTIDA, S., OHNISHI, T., & YANAGISAWA, T. (1951). The apyrase activity and adenosine-triphosphate content of the organizer region of *Bufo vulgaris formosus*. *Annot. zool. Jap.* **24**, 115–19.

GALE, E. F., & FOLKES, J. P. (1955). Promotion of incorporation of amino-acids by specific di- and tri-nucleotides. *Nature, Lond.* **175**, 592–3.

GREENSTEIN, J. P., & EDSALL, J. T. (1940). The effect of denaturing agents on myosin. I. Sulphydryl groups as estimated by porphyrindin titration. *Jour. biol. Chem.* **133,** 397–408.

GREGG, J. R., & BALLENTINE, R. (1946). Nitrogen metabolism of *Rana pipiens* during embryonic development. *J. exp. Zool.* **103,** 143–68.

—— & ORNSTEIN, N. (1953). Explant systems and the reactions of gastrulating amphibians to metabolic poisons. *Biol. Bull. Wood's Hole,* **105,** 466–76.

HARRIS, D. L. (1946). Phosphoprotein phosphatase, a new enzyme from the frog egg. *Jour. biol. Chem.* **165,** 541–50.

KUTSKY, P. B. (1950). Phosphate metabolism in the early development of *Rana pipiens. Jour. exp. Zool.* **115,** 429–60.

MAZIA, D., & DAN, K. (1952). The isolation and biochemical characterization of the mitotic apparatus of dividing cells. *Proc. nat. Acad. Sci. Wash.* **38,** 826–38.

NORMAN, C. (1954). Quantitative study of distribution of sulphydryl groups in the developing grasshopper (*Melanoplus differentialis*) embryo. *Physiol. Zoöl.* **27,** 141–56.

RAPKINE, L., & BRACHET, J. (1951). Recherches sur le rôle des groupes sulfhydriles dans la morphogénèse. 1. Action des inhibiteurs des groupes -SH sur l'œuf entier et sur des explantats dorsaux et ventraux chez les amphibiens. Implantation de protéines sulfhydrilées. *Bull. Soc. Chim. biol. Paris,* **33,** 427–38.

RUDNICK, D., MELA, P., & WAELSCH, H. (1954). Enzymes of glutamine metabolism in the developing chick embryo. A study of glutamotransferase and glutamine synthetase. *J. exp. Zool.* **126,** 297–322.

SHUMWAY, W. (1940). Stages in the normal development of *Rana pipiens. Anat. Rec.* **78,** 139–47.

SZE, L. C. (1953). Respiration of the parts of the *Rana pipiens* gastrula. *Physiol. Zoöl.* **26,** 212–23.

WAELSCH, H. (1952*a*). Certain aspects of intermediary metabolism of glutamine, asparagine and glutathione. *Advanc. Enzymol.* **13,** 237–319.

—— (1952*b*). The biological significance of the gamma-glutamyl radical. *Phosphorus Metabolism,* **2,** 109–25.

(Manuscript received 27 : vi : 55)

Inhibition of Glyceraldehyde Phosphate Dehydrogenase by Salts other than Lithium Chloride

by RICHARD G. HAM[1] *and* ROBERT E. EAKIN

From the Biochemical Institute and the Department of Chemistry, the University of Texas, Austin

LALLIER (1954) has shown that 0·4 M lithium chloride strongly inactivates glyceraldehyde phosphate dehydrogenase—a finding which might partially explain some of the developmental changes found in lithium-treated embryos. In an attempt to establish an enzymatic basis for the morphological effects of lithium ion on Hydra which have been observed in this laboratory (Ham & Eakin, 1955), we have repeated the enzyme study with lithium chloride and extended it to include a number of other salts as controls.

TABLE 1

Effects of various salts on glyceraldehyde phosphate dehydrogenase

Salt	*(Moles/L.)*	*DPNH⁺ formed (moles × 10^{-12}/cm.³/sec.)*		*Activity remaining (%)*
		Before addition	*After addition*	
None	—	155	—	100
LiCl	0·2	174	88	51
	0·4	155	35	23
	0·8	161	5*	3
KCl	0·2	139	54	39
	0·4	155	24	15
NaCl	0·4	148	21	14
NaSCN	0·08	126	32	25
	0·4	150	0	0
$NaNO_3$	0·4	161	11†	7
NaAcetate	0·4	152	75	49
LiAcetate	0·4	131	38	29

* Initial rate after addition; 2 minutes later rate had fallen to 0.
† Initial rate after addition; 4 minutes later rate had fallen to 0.

From typical data (Table 1), it is obvious that the inhibition of glyceraldehyde phosphate dehydrogenase activity is in no way a specific effect due to lithium ions. Both sodium chloride and potassium chloride produced a greater inhibition

[1] National Science Foundation Predoctoral Fellow.

Authors' address: The University of Texas, Austin, Texas, U.S.A.

[J. Embryol. exp. Morph. Vol. 4, Part 1, pp. 93–95, March 1956]

than did lithium chloride. From the various sodium salts tested, it was found that the anion may be of more importance than the cation in determining the degree of inhibition, although the cation also has some effect. It is of particular interest to note that sodium thiocyanate, which reverses the effect of lithium ions in many biological systems, is five times as effective as lithium chloride in inhibiting the enzyme system.

It would appear that the glyceraldehyde phosphate dehydrogenase enzyme system is of no value for studying the biochemical mechanisms leading to the morphological effects of lithium salts.

EXPERIMENTAL METHOD

Glyceraldehyde phosphate dehydrogenase activity was measured by spectrophotometric determination of reduced diphosphopyridine nucleotide (DPN) at 340 mμ (Cori, Slein, & Cori, 1948; Verlick, 1955). A zero order (enzyme limiting) system was used which gave a constant rate of reaction for the first 6 minutes and which deviated only very slightly by 10 minutes. The reactions were carried out in a 1 cm. silica spectrophotometer cuvette containing a final volume of 3·6 c.c. The final composition of the reaction mixture was: disodium arsenate, 0·011 M; sodium pyrophosphate, 0·0028 M; DPN, O·0004 M; glyceraldehyde 3-phosphate (prepared enzymatically from fructose 1,6-diphosphate and used without purification), 0·0014 M; and a crystalline preparation of glyceraldehyde phosphate dehydrogenase (rabbit muscle: Worthington Biochemical Company), approximately 6 mg. per cuvette. The reaction mixture was buffered at pH 8·5–8·7. Reaction was initiated by addition of substrate after the other components had been allowed to stand at least 15 minutes at room temperature to assure full activation of the enzyme. The substrate was prepared by incubating 0·010 M fructose 1,6-diphosphate with crystalline aldolase (Nutritional Biochemical Company) for 3 hours at 37° C.

Since the glyceraldehyde phosphate dehydrogenase was added in a very small volume (0·05 c.c.), considerable variations occurred from test to test. Errors due to these variations were eliminated by allowing each system to serve as its own control. The reaction was allowed to proceed exactly 2 minutes before appropriate amounts of 6 M solutions of the test salts were added. The reaction rate was then followed for another 8 minutes. The degree of inhibition was then determined from the rates before and after addition. Optical densities were read every 20 seconds, and converted to moles of reduced DPN using $a = 6{\cdot}22 \times 10^6$ cm.^{2} moles^{-1} (Kornberg & Horecker, 1953). Except as noted the rates of reaction after addition of salt solutions were also constant.

SUMMARY

1. The previously reported inhibition of glyceraldehyde phosphate dehydrogenase by 0·4 M lithium chloride was confirmed.

2. A number of salts of other cations were found to give comparable or greater inhibition than lithium chloride.

3. Sodium thiocyanate, a biological antagonist of lithium ion, was found to be five times as active as lithium chloride in inhibiting the enzyme.

4. It was concluded that the inhibition of glyceraldehyde phosphate dehydrogenase by lithium chloride does not constitute a suitable system for the study of biochemical mechanisms leading to lithium-induced morphological changes.

REFERENCES

CORI, G. T., SLEIN, M. W., & CORI, C. F. (1948). Crystalline d-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle. *J. biol. Chem.* **173,** 605–18.

HAM, R. G., & EAKIN, R. E. (1955). Unpublished observations.

KORNBERG, A., & HORECKER, B. L. (1953). Diphosphopyridine nucleotide. *Biochemical Preparations* (E. E. Snell, Editor), **3,** 20–24. New York: John Wiley & Sons.

LALLIER, R. (1954). Chlorure de lithium et biochimie du développement de l'œuf d'Amphibien. *J. Embryol. exp. Morphol.* **2,** 323–39.

VELICK, S. F. (1955). Glyceraldehyde-3-phosphate dehydrogenase from muscle. *Methods in Enzymology* (Colowick, S. P., & Kaplan, N. O., Editors), **1,** 401–6. New York: Academic Press.

(Manuscript received 18: viii: 55)

INSTRUCTIONS FOR CONTRIBUTORS

Contributions and correspondence about them should be sent to Mr. M. Abercrombie, Department of Anatomy and Embryology, University College, Gower Street, London, W.C.1, England.

Contributions should be as concise as possible. They should be typewritten, double-spaced, on one side of the paper, the pages numbered. They should be fully ready for press, since revision in proof will not be possible. Footnotes should be avoided. The author is advised to keep a copy of the typescript.

Tables should be on separate sheets, and numbered. Authors should indicate the places for their insertion in the margin of the typescript.

The Summary should be in numbered paragraphs, and will be put at the end of the text. It should not exceed 500 words.

Acknowledgements should be placed immediately before the list of references.

References should be listed alphabetically. In the text they should be referred to by the author's name and the year of publication. If more than one reference to the same author and year is quoted, use suffixes *a*, *b*, &c. (e.g. 1951*a*) in text and reference list. Papers should be listed as follows: Surname followed by initials of author(s); year of publication; title of paper; name of Journal (World List of Scientific Periodicals abbreviation), underlined; volume number; first and last page: e.g. Spemann, H., & Mangold, H. (1924). Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. Arch. mikr. Anat. **100**, 599–683. Books should be listed as follows: Surname followed by initials of author(s); year of publication; title underlined; place of publication; publisher: e.g. Dalcq, A. (1941). L'Œuf et son dynamisme organisateur. Paris: Albin Michel.

Illustrations. Line drawings should be used wherever possible, in preference to illustrations requiring half-tone reproduction. In general their size should be such that they are reduced for publication to approximately two-thirds linearly. When reduced, no illustration together with its lettering should exceed five inches in width, and, if it is to occupy a whole page, seven and a half inches in length. Name of author and figure number should be written on the back, together with the proposed reduction in size. Magnification should be indicated by a scale-line drawn in Indian ink on or beside all drawings and photographs.

Black and white line or line and dot drawings should have all lettering or numerals put in lightly in block capitals with a soft pencil. Charts and graphs may be drawn in Indian ink on graph paper ruled in pale blue. Where several drawings are grouped as one figure, distinguish them by capital letters (in pencil). Legends for all line drawings should be typed together on a separate sheet. The places for insertion in the text should be indicated in the margin of the typescript. The drawings should be referred to in the text as 'Text-fig. 1', etc.

Half-tone illustrations (photographs or drawings other than black and white) will be limited in number; authors may be asked to provide a grant for an excessive number. If fine detail is not required they can be reproduced amongst the text (please indicate position), in which case they should be numbered in series with the line drawings, if any. If fine detail is required, they will be on a separate plate. The legends should then be typed on a separate sheet, headed 'Explanation of Plate(s)', and the illustrations should be referred to in the text as 'Plate 1, fig. A', &c. Leader lines should be drawn in Indian ink. *All* letters or numerals which are to go *on* the illustration, should be in pencil (block capitals) on a covering sheet of transparent paper. Lettering or numerals to go at the side should be written there in pencil (block capitals); photographs, for this purpose, should be mounted on card. If the author wishes the block-maker to pay special attention to reproducing any part of an illustration, he should indicate this on the transparent paper. Photographs should be glossy bromide prints.

Proofs. Authors will receive one set of proofs for correction and return to the Editor. Authors may be required to pay for an excessive number of alterations in proof, apart from printer's errors.

Reprints. Authors will receive fifty free reprints. Additional copies may be ordered for purchase when proofs are returned to the Editor.

Journal of
Embryology and Experimental Morphology

[J. Embryol. exp. Morph.]

VOLUME 4 *March 1956* PART 1

Contents

PRINTED IN GREAT BRITAIN, AT THE UNIVERSITY PRESS, OXFORD
BY CHARLES BATEY, PRINTER TO THE UNIVERSITY